UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

JUSCELINO DE FREITAS JARDIM

ESTUDO CLINICOPATOLÓGICO DA ANGIOGÊNESE, LINFANGIOGÊNESE E DENSIDADE DE CÉLULAS DENDRÍTICAS EM CARCINOMAS DE CÉLULAS ESCAMOSAS DE LÍNGUA E ASSOALHO BUCAL

CLINICOPATHOLOGICAL STUDY OF ANGIOGENESIS, LYMPHANGIOGENESIS AND DENDRITIC CELL DENSITY IN SQUAMOUS CELL CARCINOMA OF THE TONGUE AND FLOOR OF THE MOUTH

PIRACICABA
2017

JUSCELINO DE FREITAS JARDIM

ESTUDO CLINICOPATOLÓGICO DA ANGIOGÊNESE, LINFANGIOGÊNESE E DENSIDADE DE CÉLULAS DENDRÍTICAS EM CARCINOMAS DE CÉLULAS ESCAMOSAS DE LÍNGUA E ASSOALHO BUCAL

CLINICOPATHOLOGICAL STUDY OF ANGIOGENESIS, LYMPHANGIOGENESIS AND DENDRITIC CELL DENSITY IN SQUAMOUS CELL CARCINOMA OF THE TONGUE AND FLOOR OF THE MOUTH

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutor em Estomatopatologia, área de Patologia

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements to obtained the degree of Doctor in Estomatopatologia, in Pathology area.

Orientador: Prof. Dr. Luiz Paulo Kowalski

Este exemplar corresponde à versão final da tese defendida pelo aluno Juscelino de Freitas Jardim, orientada pelo prof. Dr. Luiz Paulo Kowalski.

PIRACICABA

2017

Agência(s) de fomento e nº(s) de processo(s): CNPq

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

J284e

Jardim, Juscelino de Freitas, 1989-
Estudo clinicopatológico da angiogênese, linfangiogênese e densidade de células dendríticas em carcinomas de células escamosas de língua e assoalho bucal / Juscelino de Freitas Jardim. – Piracicaba, SP : [s.n.], 2017.

Orientador: Luiz Paulo Kowalski.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Carcinoma de células escamosas. 2. Marcadores biológicos. 3. Neoplasias bucais. I. Kowalski, Luiz Paulo. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Clinicopathological study of angiogenesis, lymphangiogenesis and dendritic cell density in squamous cell carcinoma of the tongue and floor of the mouth
Palavras-chave em inglês:
Carcinoma, squamous cell
Biological markers
Mouth neoplasms
Área de concentração: Patologia
Titulação: Doutor em Estomatopatologia
Banca examinadora:
Luiz Paulo Kowalski [Orientador]
Fábio de Abreu Alves
Claúdia Malheiros Coutinho Camillo
Pablo Agustin Vargas
Alan Roger dos Santos Silva
Data de defesa: 17-03-2017
Programa de Pós-Graduação: Estomatopatologia

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 17 de Março de 2017, considerou o candidato JUSCELINO DE FREITAS JARDIM aprovado.

PROF. DR. LUIZ PAULO KOWALSKI

PROF. DR. FÁBIO DE ABREU ALVES

PROFª. DRª. CLÁUDIA MALHEIROS COUTINHO CAMILLO

PROF. DR. PABLO AGUSTIN VARGAS

PROF. DR. ALAN ROGER DOS SANTOS SILVA

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

DEDICATÓRIA

Este trabalho é dedicado aos meus pais, **Antonio Celino Holanda Jardim** e **Delfina Lopes de Freitas Jardim**. Por serem meus maiores mestres e sempre me incentivarem a lutar pelos meus sonhos.

AGRADECIMENTOS

A **Deus**, sabendo que nada sou, agradeço a Deus Pai pela inspiração e força que me permitiram concretizar mais este trabalho. Obrigado Senhor por ter sido minha inesgotável fonte de força e me ouvir nos lamentos noturnos por meus atos insólitos. Por ter me confortado em momentos que eu necessitei e me conceder paz de espírito e sua misericórdia infinita.

Aos meus pais **Antonio Celino Holanda Jardim** e **Delfina Lopes de Freitas Jardim** que pelo amor e exemplos de vidas sempre me ensinaram a lutar pelos meus ideais e buscar meus sonhos. Agradeço por todo o apoio dado neste período que estive distante de vocês, bem como carinho, compreensão e paciência.

Ao meu orientador **Prof. Luiz Paulo Kowalski**, pela oportunidade e confiança para a execução deste trabalho. Embora nunca tenha me expressado diretamente, é uma grande honra ser orientado pelo senhor. Agradeço também pela orientação segura, transmissão de conhecimentos e de ideias.

Aos meus irmãos **Celino Júnior, Jussara Freitas e Juliano Freitas** pelo carinho, amor e apoio constantes para que eu buscasse meus objetivos, mesmo que isso tenha significado nosso distanciamento temporário.

Á **Ana Lucia Francisco**, pela amizade construída, conversas, companheirismo e compartilhamentos de emoções, além da inquestionável e valiosa ajuda na execução deste trabalho.

Ao amigo **Rogerio Gondak,** pela contribuição valorosa neste trabalho e em outros trabalhos. Pela amizade construída, pelas conversas, pelos seus relatos de experiência. Sinto-me muito satisfeito de poder contar com uma pessoa tão qualificada e competente como você.

Ao Prof. **Alan Roger dos Santos Silva**, pela atenção dispendida, pelos ensinamentos transmitidos, discussão de trabalhos e amizade. Deixo externado aqui minha profunda admiração pessoal.

À **Fernando Mariano**, uma pessoa com grandes qualidades, e sempre disposta a ajudar. Deixo aqui registrado meu agradecimento pelas conversas e ajuda que me deste desde minha chegada em Piracicaba.

Ao Professor **Pablo Agustin Vargas,** exímio conhecedor da patologia humana. Agradeço pelos ensinamentos transmitidos nesta área.

Ao Dr. **Fábio de Abreu Alves,** pela disponibilidade e abertura do departamento de Estomatologia do AC Camargo Cancer Center para que eu acompanhasse as atividades desempenhadas.

Ao Prof. **Oslei Paes de Almeida**, referência mundial na área de Patologia Oral, pelo convite para participar de seu grupo de discussão, e pela atenção sempre dada enquanto estive em Piracicaba.

À **Universidade Estadual de Campinas** na pessoa de seu magnífico reitor **Prof. Dr. José Tadeu Jorge** e à sua **Faculdade de Odontologia,** na pessoa de seu diretor **Prof. Dr. Guilherme Elias Pessanha Henriques**, por oferecer-me suas instalações e quadro de Pessoal que ocupam posições de destaque no cenário nacional e internacional.

Ao **Programa de Pós-Graduação em Estomatopatologia** na pessoa de seu coordenador **Prof. Dr. Márcio Ajudarte Lopes,** e a todos os alunos e professores que o compõe. Agradeço pelo espaço científico ofertado para realização das atividades acadêmicas e pelas amizades conquistadas

À **Fundação Antonio Prudente**, na pessoa de seu presidente **Dr. José Hermilio Curado,** por permitir que alunos oriundos de outras instituições possam usufruir das instalações do A.C. Camargo Cancer Center, conduta que me beneficiou durante a realização de meus experimentos.

Ao **A.C. Camargo Cancer Center**, na pessoa do Diretor do Departamento de Otorrinolaringologia e Cabeça e Pescoço **Dr. Luiz Paulo Kowalski**, pela receptividade e espaço para execução de meu trabalho nesta instituição.

Ao Prof. Dr. **Fernando Augusto Soares,** por permitir minha entrada ao parque de instalações do departamento de Patologia do A.C. Camargo Cancer Center.

À **Lucas de Oliveira**, pela incrível que pessoa que é, e por saber que posso sempre contar com você.

Ao amigo **Cosmo Helder**, exemplo de humildade e beleza da docência. Meu agradecimento por sempre poder contar com sua pessoa.

À professora **Karuza Alves**, minha sempre fonte de inspiração. É sempre uma honra chegar um passo mais próximo daqueles que admiramos.

Ao grande amigo **José Flávio Sobral**. Agradeço por cada conversa, discussão nas áreas de política, economia, geografia, viagens, dentre outros. Contar com você é uma grande alegria para mim.

Aos amigos **Wesley Paulino, Ruan Faquin e Manuel Lima**, por toda a amizade construída durante o período de pós-graduação.

Ao amigo **Luiz Filipe Barbosa Martins**, aluno de doutorado do programa de Odontopediatria da FOP-UNICAMP. Pelas conversas, apoio, discussão de temas diversos, diversões e amparos em momentos tristes. Obrigado pela amizade verdadeira e gratuita.

Ao amigo **Tallis Maia**, exemplo de pessoa que vale a pena estar próximo e por toda a força nestes momentos que antecederam a defesa desta tese.

Às amigas de Quixadá, **Thayla Hellen e Rafaela Costa**, que estão cursando suas pós-graduações em Piracicaba. Por sempre estarem na torcida e dando forças a qualquer momento.

Ao grande amigo **Gabriel Viana,** por ter estado presente em diversos momentos desta estadia longe de casa. Um grande amigo que levarei para toda a vida.

À **Everardo Fonseca,** pela grande amizade construída ao longo dos anos, e por fazer questão de estar presente no dia da defesa desta tese.

Aos técnicos de Laboratório do A.C. Camargo **Sueli, Carlinhos e Severino**, por toda a ajuda durante a fase laboratorial deste trabalho.

À **Drª. Cláudia Coutinho** por sempre se colocar à disposição e pela ajuda durante a execução da Imunoistoquímica em meu estágio no Centro Internacional de Pesquisas (CIPE).

Ao **Conselho Nacional de Desenvolvimento Tecnológico e Científico (CNPq)** pela bolsa concedida.

Por fim, se por falha da minha memória, cometi alguma injustiça e esqueci de mencionar algum nome, deixo minhas desculpas agradecendo a todos que de alguma forma me acompanharam e me ajudaram neste importante passo da minha vida.

EPÍGRAFE

"A persistência é o menor caminho do êxito"

(Charles Chaplin)

RESUMO

O carcinoma de células escamosas, também chamado carcinoma espinocelular (CEC) é a neoplasia maligna mais frequente na cavidade bucal, correspondendo a quase 95% destas lesões, e cerca de 38% dos tumores malignos de cabeça e pescoço. Mais de 50% dos portadores deste tipo de tumor apresentam estágio avançado da doença no momento do diagnóstico, fator que reflete em baixas taxas de sobrevidas em 5 anos. A relação de densidades de vasos linfáticos e sanguíneos, assim como a densidade de células dendríticas em CECs deveriam apresentar um papel importante na progressão, disseminação e metástase, entretanto estes mecanismos e seus impactos prognósticos ainda não são bem compreendidos. O propósito do primeiro estudo consistiu em avaliar a correlação entre as densidades vasculares sanguíneas e linfáticas com dados prognósticos em pacientes com CEC em estádio clínico avançado. Reações de imuno-histoquímica para os marcadores D240, CD34 e CD105 foram executadas em 88 casos de CECs de língua e assoalho de boca em estádio clínico avançado. A densidade vascular linfática (DVL), densidade vascular sanguínea (DVS) e a densidade de vasos neoformados (DVN) foram avaliados através de contagem do número de vasos imunomarcados em 4 áreas de "hotspots" para cada marcador, tanto intratumoral quanto peritumoral em aumento de 40x. Os dados obtidos foram correlacionados com parâmetros clínicos e patológicos e de sobrevida. Alta DVL intratumoral foi associada com a presença de rutura de capsula nodal (p= 0,03), enquanto DVN peritumoral foi correlacionada com envolvimento linfonodal (p= 0,05). Recorrência foi associada com elevadas DVL intratumoral (p< 0,0001), DVS intratumoral (p= 0,036) e com DVN peritumoral (p= 0,047). Para sobrevida global em 5 anos houve associação com altas contagens de DVL (p= 0,0016) DVN (p= 0,009) intratumorais. Ainda, DVL iintratumoral foi um fator independente para sobrevida livre de doença e sobrevida global baseado no modelo Cox proportional hazard. No segundo estudo, realizou-se reações de imuno-histoquímica para os marcadores CD1a e CD83 em 53 casos de CECs de língua oral e assoalho de boca, objetivando correlacionar a densidade de células dendríticas com o prognóstico e taxas de sobrevida dos pacientes. Foi demonstrado que a a diminuição da densidade de CD1a+ em estroma teve associação com metástase linfonodal (p = 0,05), enquanto contagens menores de CD83+ estromais foram correlacionadas com histórico de fumo (p = 0,04), metástase linfonodal (p = 0,015) e rutura de cápsula nodal

(*p* = 0,018). Resultados originados de análise de sobrevivência e recorrência da doença obtidas pelo modelo de Cox proportional hazard demonstraram que a diminuição do número de células CD1a+ no estroma é fator independente tanto para sobrevida global quanto para sobrevida livre de doença. Em conclusão nossos resultados sugerem que altas contagens de DVL intratumorais, bem como diminuição do número de células CD1a+ em estroma têm forte impacto na sobrevida dos pacientes com CECs de língua e assoalho de boca.

Palavras-chave: carcinoma de células escamosas, biomarcadores, fatores prognósticos, neoplasia, boca.

ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common malignancy in the oral cavity, accounting for almost 95% of these injuries, and about 38% of malignant tumors of the head and neck. More than 50% of all patients have advanced disease at the time of diagnosis, factor that reflects in low survival rates at 5 years. The presence and density of lymphatic vessels and blood vessels, as well as dendritic cells density, in OSCC should play a key role in the progression, dissemination and metastasis, however this mechanisms and prognostic impact are not so well understood. The first study aimed to evaluate the correlation between the lymphatic and blood vessel densities and correlate with the prognostic outcomes in advanced stage OSCC. Immunohistochemical reactions for D240, CD34 and CD105 were performed in 88 advanced stage OSCC cases of the oral tongue and floor of the mouth. The lymphatic vascular density (LDV), blood vascular density (BVD) and neoformed vascular density (NVD) were assessed by counting positive reactions in 4 hotspot areas for each marker, both intratumoral (IT) and peritumoral (PT) at high magnification (x40). These data underwent correlation with clinicopathological and survival outcomes. High density of IT LVD was associated with the presence of extracapsular spread of lymph node metastasis (p= 0.03), while PT NVD was correlated with pathological nodal status (p= 0.05). No associations were found among BVD with any of the clinicopathologic parameters analyzed. Recurrence were correlated with IT LVD (p< 0.0001), IT BVD (p= 0.036) and IT NVD (p= 0.047). Overall survival rates in 5 years were associated with the high IT LVD (p= 0.0016) and IT NDV (p= 0.009). Yet, IT LVD was an independent factor for disease-free survival and for overall survival based on the Cox proportional hazard model. In the second study, we performed immunohistochemical reactions for CD1a and CD83 cells in 53 cases of OSCC of the oral tongue and flor of the mouth aiming to correlate the density of dendritic cells with prognostic and survival outcomes. We have demonstrated that the diminished density of stromal CD1a+ had association with lymph node metastasis (p = 0.05), whereas low counts of stromal CD83+ were correlated with smoking history (p = 0.04), lymph node metastasis (p = 0.015) and extracapsular spread of lymph nodes (p = 0.018). Results from the survival analysis using the Cox proportional hazard model has shown that decreased number of stromal CD1a+ is an independent factor for overall survival and disease-free survival. In conclusion, our results suggest that high counts of intratumoral LVD, as

well as the decresead number of stromal CD1a+ have strong impact on survival outcomes in OSCC of oral tongue and floor of the mouth.

.

Keywords: squamous cell carcinoma, prognosis, biomarkers, cancer, oral cavity.

SUMÁRIO

1 INTRODUÇÃO

A Organização Mundial de Saúde (2005) define câncer como um grupo de doenças que podem afetar qualquer parte do corpo, sendo caracterizado pela rápida progressão de células anormais que crescem além de seus limites habituais, e que podem então invadir estruturas adjacentes e metastatizar para outros órgãos.

É inquestionável que o câncer é um problema de saúde pública, especialmente entre os países em desenvolvimento, onde é esperado que, nas próximas décadas, o impacto do câncer na população corresponda a 80% dos mais de 20 milhões de casos novos estimados para 2025 (Ministério da Saúde 2015).

O Instituto Nacional do Câncer (INCA) estimou, para o ano de 2016, aproximadamente 600.000 casos novos de câncer no Brasil. Desse total, 15.490 (2,58%) acometeriam a cavidade oral (boca e orofaringe), sendo o quinto tipo mais frequente no gênero masculino e o décimo segundo no feminino. Esses valores correspondem a um risco estimado de 11,27 casos novos a cada 100 mil homens e 4,21 a cada 100 mil mulheres (Ministério da Saúde 2015).

A carcinogênese oral é um processo multifásico que requer a desestabilização de vários sistemas que controlam e coordenam o comportamento e homeostase celular (Todd et al., 2002), havendo ruptura da sinalização celular, reparo do DNA e alteração do ciclo celular (Bettendorf et al., 2004). Segundo Boshoff e Weiss (2001), ocorre na maioria dos casos, um acúmulo sequencial de mutações somáticas por muitos anos, antes da expansão clonal de células malignas.

O tipo histológico mais comum de câncer bucal é o carcinoma de células escamosas, também chamado carcinoma epidermóide ou carcinoma espinocelular (CEC) pelo fato da proliferação celular originar-se da camada espinhosa do epitélio. Corresponde a 95% de todos os tumores malignos da boca, cerca de 38% dos tumores malignos situados na região de cabeça e pescoço (Scully, 2009).

O desenvolvimento do CEC relaciona-se diretamente a alterações na estrutura e regulação genética, principalmente naqueles genes reguladores do processo de divisão celular (Peltonen et al., 2010). Pich et al. (2004) citaram que dentre as alterações, as mutações do gene P53 e Ki67 são as mais comuns, podendo ser utilizadas como indicadores de prognóstico nos pacientes com carcinomas de boca. Alterações como perda de heterozigose, instabilidade de microsatélites e

repetições de sequências também contribuem para a ocorrência de tais alterações genéticas (Kodani et al., 2001).

Já está bem estabelecido que o câncer de boca possui etiologia multifatorial, fazendo parte tanto fatores intrínsecos quanto extrínsecos, e sendo necessário a ação de mais de um fator desencadeante para a produção de malignidade (Scully, 2009; Nagpal et al., 2003). Tabagismo e o etilismo crônico são os principais fatores de risco, uma vez que apenas 15 a 20% dos portadores de lesões malignas bucais não tem história pregressa destes hábitos (Warnakulasuriya, 2009; Bettendorf et al., 2004). Ainda, o risco de desenvolvimento desta neoplasia na população geral é aumentada em cerca de sete vezes com o tabagismo, e em até 15 vezes quando este hábito está associado ao consumo crônico de álcool (Kruse et al., 2010). Fatores intrínsecos tais como alterações genéticas, deficiências nutricionais e imunossupressão; e fatores extrínsecos como radiação solar e alguns vírus também são considerados agentes etiológicos da doença (Nagpal et al. 2003).

A família do Papiloma Vírus Humano (HPV) conta com mais de 100 subtipos virais catalogados (van Monsjou et al., 2013), e sua infecção está presente em aproximadamente 5,2% de pacientes com câncer no mundo inteiro, incluindo tumores de ânus, trato genital e nos últimos anos tem ganhado destaque pela relação de casos localizados na orofaringe (Chung et al., 2013) sendo a exposição, particularmente, aos subtipos 16 e 18 bem reconhecidos nestes casos.

Os portadores de CEC oral são especialmente pacientes do gênero masculino entre a 5º e a 8º décadas de vida. No entanto, observa-se atualmente um aumento na incidência desta neoplasia em indivíduos jovens, com menos de 45 anos de idade e que muitas vezes não foram expostos aos fatores de risco ambientais mais significativos (tabaco e álcool) (Warnakulasuriya, 2009).

O tumor tem apresentação clínica variada, apresentando-se clinicamente de acordo com o tempo de evolução da doença. Lesões iniciais podem se apresentar como leucoplasias, eritroleucoplasias ou úlceras. A neoplasia pode se apresentar com aspecto exofítico ou endofítico, mostrando às vezes aparência nodular. Língua (borda lateral) e assoalho bucal são as localizações bucais mais comuns de acometimento (Scully, 2009).

As neoplasias malignas assim com o CEC, exibem caracteristicamente um crescimento infiltrativo, sendo capazes de invadir tecidos vizinhos, ganhar uma via de disseminação, chegar a sítios distantes (metástases) (Takes et al., 2012). Tal

fenômeno é um processo complexo e é considerado um evento tardio na carcinogênese que se inicia com a proliferação celular, havendo posteriormente perda do contato com células vizinhas, migração através da matriz intersticial, invasão de vasos linfáticos e sanguíneos e crescimento em linfonodos e órgãos distantes. Dentre as localizações anatômicas o câncer de língua apresenta um alto potencial de invasão, e grande probabilidade de desenvolver metástase para linfonodos regionais (Kowalski *et al*., 1993).

A agressividade destes tumores está relacionada a diversos fatores, dentre os quais cita-se o grau histológico de malignidade, tamanho da lesão, grau de comprometimento dos tecidos vizinhos, presença de metástase no momento do diagnóstico e localização anatômica do tumor (Woolgar, 2007; Massano et al., 2006). Parâmetros clínicos e imaginológicos como tamanho tumoral e disseminação metastática, consistem em excelentes indicadores prognósticos do paciente. Estes parâmetros permitem o estadiamento das neoplasias malignas através do sistema TMN, onde T se refere ao tamanho do tumor (variando de T1 a T4), N à propagação aos linfonodos regionais (N0 a N3) e M à metástase à distância (M0 e M1). De acordo com os parâmetros estabelecidos pode-se classificar as lesões em estádios clínicos de I a IV (UICC*,* 2009). O prognóstico do câncer oral se mostra variável, com taxas de sobrevida de 5 anos, oscilando entre 74%, para lesões iniciais, e 29%, para carcinomas diagnosticados em estádio IV (Carvalho et al., 2004).

Segundo Woolgar (2006) a avaliação do espécime cirúrgico fornece importantes informações a respeito da agressividade do tumor, e que implicam diretamente com o prognóstico dos pacientes. Os principais fatores prognósticos reconhecidos são: dediferenciação, invasão de estruturas, ruptura linfonodal, invasão linfática e vascular e invasão perineural.

Um dos maiores desafios na pesquisa em câncer é o desenvolvimento de terapia capaz de impedir a disseminação tumoral e metástase, pois estes eventos diminuem drasticamente as chances de cura e sobrevida dos pacientes. A maioria dos tumores sólidos apresenta metástase para os linfonodos regionais, preferencialmente via vasos linfáticos, que geralmente é o primeiro sinal de disseminação cancerígena (Takes et al., 2012). No entanto, tumores malignos também têm a habilidade de induzir o crescimento de novos vasos sanguíneos a partir de vasos periféricos (angiogênese), que são importantes para a progressão tumoral, crescimento, agressividade e habilidade para produzir metástases (Folkman, 1995; Massano et al., 2006). A

estrutura desorganizada e tortuosa dos neovasos permite a invasão por células malignas. Ramificações dos vasos pré-existentes comunicam com a rede vascular imatura possibilitando a migração destas células. Estas seguem, assim na circulação sanguínea, podendo originar metástases à distância.

Para que a angiogênese ocorra, as células tumorais expressam fatores de crescimento angiogênicos. Dentre os mais importantes desta classe, está o fator de crescimento vascular endotelial (vascular endothelial growth factor - VEGF), conhecido por estimular a proliferação das células endoteliais. Ainda, existem pelo menos seis membros da família VEGF (VEGF-A, -B, -C, -D e - E) (Cortesina & Martone, 2006). A endoglina (CD105) é uma glicoproteina expressa no endotélio angiogênico peritumoral e intratumoral e a sua ação está relacionada à ativação da proliferação endotelial (Fonsatii et al., 2003). Segundo Schimming et al. (2004) e Nagatsuka et al. (2005), a endoglina é o marcador endotelial com melhor especificidade para avaliação do padrão da angiogênese em carcinomas de células escamosas bucais, pois esta glicoproteina está envolvida no processo de indução da neovascularização. Deste modo, esta característica permite uma melhor avaliação do estado proliferativo da vasculatura tumoral.

Schimming et al. (2004) analisaram a expressão do fator de crescimento endotelial vascular (VEGF) e o marcador vascular de neo-angiogênese CD105 em 51 Carcinomas espinocelulares de boca. A quantificação da expressão do VEGF não mostrou relação estatisticamente significativa com o estadiamento TNM dos tumores. No entanto a marcação da CD105 apresentou correlação significativa com o estadiamento TNM, onde os tumores com estadiamento T1 possuíam uma menor taxa de neovascularização do que os tumores com os estadiamentos T2, T3 e T4.

A invasão linfovascular é definida por Sutton et al. (2003) como a presença de agregados de células tumorais no interior de vasos linfáticos ou infiltrando seu endotélio. A disseminação de células tumorais via vasos linfáticos e suas implicações no tratamento e prognóstico dos pacientes tem sido estudadas há anos (Jones et al. 2009). As características próprias dos capilares linfáticos, como a descontinuidade da membrana basal, facilitam a permeação de células malignas. Outra característica dos vasos linfáticos como a baixa velocidade do fluxo linfático e a composição da linfa (semelhante à do fluido intersticial) favorecem a viabilidade celular. Como tal, a corrente linfática é preferencial para a disseminação de muitos tumores malignos, principalmente os que apresentam histogênese epitelial.

Anticorpo D2-40 detecta um marcador seletivo para endotélio linfático, permitindo a identificação dos vasos linfáticos em tecidos fixados em formalina e incorporados em parafina e diferenciando-os dos vasos sanguíneos (Xuan *et al*., 2005; Miyahara *et al*. 2007). A densidade linfática, usando o anticorpo D2-40 para detectar vasos linfáticos, foi significativamente correlacionada a metástase linfonodal ($p<0.001$) em carcinoma epidermóide de boca no estudo de Sugiura et al. (2009) e foi um fator de risco independente para metástase linfonodal em carcinomas de língua no estudo de Zhang et al. (2011).

Um elevado índice de densidade linfática (LVD, do inglês, *lymphatic vessel density*) e densidade de microvasos (MVD, do inglês, *microvessel density*) foram relacionados com metástase linfonodal e baixa taxa de sobrevida. Pacientes com elevados valores de densidade linfática pode ter um risco aumentado de metástase linfonodal, corrobando com a idéia que a LVD é responsável pela predominância de disseminação linfática no câncer de boca. (Miyahara et al*.* 2007).

O CD34 (CD-cluster of differentiation do inglês: grupos de diferenciação) é um antígeno protéico na transmembrana de 120 kD expresso nas células progenitoras hematopoiéticas, cujo gene codificador foi mapeado no cromossomo 1q (Nikollof, 1991). É encontrado na superfície de células endoteliais, especialmente durante a angiogênese (Kuzu et al., 1992), cuja expressão pode ser observada tanto em tecido normal quanto neoplásico. Sua presença nas lesões malignas tem sido investigada na tentativa de auxiliar na formulação do prognóstico destas lesões. Vários autores têm encontrado um aumento da densidade vascular obtida por CD34 associada a um pior prognóstico em carcinoma de ovário, carcinoma de esôfago, carcinoma epitelial de glândulas salivares (Doi et al., 1999; Obemair et al., 1999)

Durante os períodos de iniciação, promoção e progressão carcinogênica, alterações no sistema imunológico tem sido observados (Bennaceur et al. 2008). As células dendríticas (CDs) tem um papel chave regulação da imunidade inata e adquirida, incluindo imunidade antitumoral. Estas células estão distribuídas pelo corpo e tem um papel central de imunidade nos tecidos não linfoides, como a pele. (Austin, 1993). As CDs imaturas são originadas de precursores na medula óssea e através da corrente sanguínea migram para os tecidos periféricos, onde são capazes de fagocitar células e antígenos tumorais (Bennaceur et al. 2008).

A relação entre células dendríticas e prognóstico tem sido relatada em malignidades humanas, contudo a relação com o CEC de boca permanece bastante

dúbia. Em um estudo realizado por Goldman et al. (1998) foi evidenciado que pacientes com CEC de boca, apresentando alta expressão CD1a+, apresentaram um melhor prognóstico, impactando sobrevida global e reduzindo taxas de recidiva.

Embora bastante estudado, o CEC oral ainda permanece com muitos aspectos não compreendidos, principalmente no que concernente ao prognóstico. Em virtude disto, nosso trabalho objetivou avaliar a influência prognóstica da expressão de marcadores relacionados com a densidade vascular (CD34), angiogênese (CD105), linfangiogênese (D240), bem como do sistema imune mediado por células dendríticas (CD1a e CD83) encontrados nos espécimes cirúrgicos de pacientes submetidos à cirurgia como tratamento inicial para CECs de língua oral e assoalho de boca.

2 ARTIGOS

2.1 Artigo 1: Prognostic significance of angiogenesis and lymphangiogenesis in advanced stage oral squamous cell carcinoma

Artigo submetido à revista *Oral Oncology*

Juscelino Freitas JARDIM[1], DDS;
Ivete Rodrigues FABELO[2], MD, PhD;
Fernando Augusto Soares[3], MD, PhD
Clovis Antonio Lopes PINTO[3], MD, PhD;
Luiz Paulo KOWALSKI[2*], MD, PhD

[1]Department of Oral Diagnosis, Piracicaba Dental School, State University of Campinas
[2]Department Head and Neck Surgery and of Otorhinolaryngology, AC Camargo Cancer Center
[3]Department of Pathology, AC Camargo Cancer Center

Corresponding author:
Prof. Dr. Luiz Paulo Kowalski
Department of Head and Neck Surgery and Otorhinolaryngology,
AC Camargo Cancer Center, Rua Professor Antonio Prudente 211, Liberdade
São Paulo-SP CEP: 01509-900
E-mail: lp_kowalski@uol.com.br

ABSTRACT

Background: The presence and density of lymphatic vessels and blood vessels in oral squamous cell carcinoma (OSCC) should play a key role in the progression, dissemination and metastasis, however this mechanisms and prognostic impact are not so well understood. This study aimed to evaluate the correlation between the lymphatic and blood vessel densities with the prognostic outcomes in advanced stage OSCC. **Patients and Methods:** Immunohistochemical reactions for D240, CD34 and CD105 were performed in 88 advanced stage OSCC cases sited at the oral tongue

and floor of the mouth. The lymphatic vascular density (LDV), blood vascular density (BVD) and neoformed vascular density (NVD) were assessed by counting positive reactions in 4 hotspot areas for each marker, both intratumoral (IT) and peritumoral (PT) at high magnification (x40). These data underwent correlation with clinicopathological and survival outcomes. **Results:** High density of IT LVD was associated with the presence of extracapsular spread of lymph node metastasis (p= 0.03), while PT NVD was correlated with pathological nodal status (p= 0.05). No associations were found among BVD with any of the clinicopathologic parameters analyzed. Recurrence rates were correlated with IT LVD (p< 0.0001), IT BVD (p= 0.036) and IT NVD (p= 0.047). Overall survival rates were associated with the high IT LVD (p= 0.0016) and IT NDV (p= 0.009). Yet, IT LVD was an independent factor for disease-free survival and for overall survival based on the Cox proportional hazard model. **Conclusion:** Our results suggest that high counts of intratumoral LVD have strong impact on survival outcomes in advanced stage OSCC.

Keywords: Microvessel density; lymphatic vessel; blood vessel; oral cancer; prognosis

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasia of the head and neck regions and accounts for more than 90% of cancers in the oral cavity(1). The overall incidence of OSCC varies considerably throughout the world with incidence peaks in Southeast Asia, parts of Central and southwest Europe (Spain, France) and Brazil, which is attributed to cultural habits that involve the consumption of tobacco and alcohol, the two most widely known risk factors for oral cavity cancer (1,2). At present, the most important prognostic factors include histological tumor grade, stage, depth of the tumor invasion, and involvement of regional lymph nodes at the time of diagnosis (3).

Tumor metastasis to regional lymph nodes via the lymphatic system represents the first step of dissemination in OSCC and is a major prognostic indicator for disease progression (4). Unfortunately, more than 50% of all patients have advanced disease at the time of diagnosis, and despite aggressive and often mutilating therapeutic regimens, overall long term survival is still of less than 50% (1,2), motivating the search for prognostic factors that can be used to improve the management of the disease in each individual patient (5).

The presence and density of lymphatic vessels and blood vessels in intratumoral and peritumoral tissues should play an important role in the progression, dissemination and metastasis of carcinomas (5,6). To develop and disseminate, the tumor requires the induction of angiogenesis from a pre-existing vascular network, which guarantees the delivery of oxygen, nutrients and growth factors (7). The immunomarker CD105 (Endoglin) is a proliferation-associated protein abundantly expressed in angiogenic endothelial cells, while the anti-CD34 antibody recognizes a cell-surface antigen of approximately 110 kDa that is expressed selectively in the vascular endothelium and human hematopoietic progenitor cells. These markers are commonly used to evaluate angiogenesis in tumors (6,8).

On the other hand, the process of lymphangiogenesis (formation of new lymphatic vessels) is thought to be crucial for cancer cells to metastasise to the regional lymph nodes (9). The D240 is a highly specific marker for lymphatic endothelial cell, not staining blood vessel endothelial cells. It is accepted as a marker for M2A antigen, also called podoplanin, that is expressed both in specific cell types of normal tissues as well as in tumoral cells, and it has been correlated with aggressiveness, metastasis and poor prognosis (10, 11). However, for OSCC, the evidence is still insufficient (6,10,12).

The purpose of this study was to examine the correlations between the lymphatic and blood vessel densities with clinicopathological features and clinical outcomes in patients with advanced stage OSCC (clinical stage III and IV).

PATIENTS AND METHODS

Paraffin-embedded tissue samples from 88 advanced stage OSCC cases of the oral tongue and floor of the mouth were obtained from the Department of Pathology at AC Camargo Cancer Center, São Paulo, Brazil

All patients were treated at the Department of Head and Neck Surgery and Otorhinolaryngology at the same institution from 1998 to 2010. The eligibility criteria included previously untreated patients, without a second primary tumor and submitted to treatment in the institution. The National Human Research Ethics Committee approved this study (Protocol # 1684/12). The medical charts and pathological reports of all patients were examined to obtain clinicopathological data (clinical stage, tumor size, histological grade, lymphovascular embolization, perineural infiltration, pattern of

invasion, margins, nodal status), including information regarding lifestyle (smoking habit and alcohol consumption), and demographic data (age, gender, and race).

The tumors were re-staged according to the 2002 version of the International Union Against Cancer TNM classification (13). The histological grade was determined on the basis of classification proposed by the World Health Organization (14).

Paraffin-embedded formalin-fixed tissue sections (4 mm) were deparaffinized in xylene and rehydrated in graded ethanol solutions to water. Thereafter, sections were treated with endogenous peroxidase quenching (0.3% H_2O_2 for 15 min) and blocked for unspecific proteins (Novolink Protein Block®, Leica), 20 min each prior to primary antibody incubation. Pressure cooker antigen retrieval consisted of one period at 125°C for 30 min and 90°C for 10 min in 10 mM citric acid solution (pH 6.0) or EDTA/TRIS (pH 9.0) followed by a washing step with phosphate-buffered saline (PBS). The incubations with the primary antibodies diluted in PBS or ready to use were conducted overnight at 4 °C: details on antigen retrieval methods as well as primary antibody clones, source, and titer are described in **Table 1**.

The sections were washed and incubated with secondary antibodies (Novolink™ Post Primary, Leica Biosystems, New Castle, UK) for 30 min followed by the polymer detection system (Novolink™ Polymer, Leica Biosystems) for 30 min at room temperature. Reactions were developed with a solution containing 0.6 mg/ml of 3,30-diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, MO) and 0.01 % H_2O_2 and then counter-stained with Mayer's hematoxylin, dehydrated and mounted with a glass coverslip. Positive controls (a tissue known to contain the antigen under study) were included in all reactions in accordance with manufacturer's protocols.

The immunohistochemical staining for CD105 was automatically performed in the Roche/Ventana (BenchMark XT) IHC/ISH Automatic Staining Module. The consecutive steps were performed with the following: (1) EZ prep deparaffinization, 16 minutes; (2) CC1, 30 minutes; (3) H_2O_2, 8 minutes; (4) incubation with antiCD105 antibody, 32 minutes; (5) secondary antibody, 8 minutes; (6) DAB, 4 minutes; (7) copper sulfate solution to enhance the DAB color, 4 minutes; (8) counterstaining with Mayer hematoxylin, 4 minutes; (9) postcounterstaining with bluing reagent, 4 minutes.

The microvessel density was obtained manually, based in the method initially described by Weidner (15). Briefly, at low magnification (10x) four "hot spots" in intratumoral and peritumoral area were localized. Subsequently counting was

performed at a high power magnification field (40x). The examination of each hotspot corresponds to a number of vessels confined to an area of 0.15mm².

The average numbers of lymphatic, blood and neoformated vessels in the four fields were converted into the number of vessels per area (mm²), and the positive reactions for CD34, CD105 and D240 were categorized, respectively, into blood vessel density (BVD), neoformed vessel density (NVD) and Lymphatic vessel density (LVD) to posterior statistical comparisons.

Analysis of the association between protein expressions and the demographic and clinicopathologic characteristics of the patients was performed using the T-student, Mann-Whitney U, Kruskal-Wallis and Pearson's chi-square tests as appropriate.

The determination of two group of observations with respect to a simple cut-point was estimate using the maximum of the standardized log-rank statistic proposed by Lausen and Schumacher, 1992 (16). We consider the maximally selected long-rank statistic for cut-points between 5% and 95% quantile of continuous measure.

Overall and disease-free survival probabilities were calculated based on the Kaplan-Meier estimator. The comparison among survival functions were assessed through of logrank test. Multivariate relative risk was evaluated by the Cox proportional hazards model. Statistical analyses were performed using using SPSS (version 23) and R software version 3.2.1 and the significance level was fixed at 5% for all statistical tests.

RESULTS

The study sample consisted of 64 males (72.7%) and 24 females (27.3%) with a mean age of 57 years (range, 27-82 years). The majority of the cases (72.7%) were seen in the oral tongue. T1 and T2 were presented in 20 (22.7%) cases, whereas T3 and T4 constituted 68 cases (77.3%). The median follow up time was of 33.4 months (range 3 to 178 months). The disease-free survival was estimated in 30% for 5 years. Overall survival at 5 years was of 38%.

Only 23 cases (26.1%) were node negative and among the cases with positive nodes, 28 (31.8%) were N1 and 37 (42.1%) N2. Most of the cases (59.1%) were TNM stage IV. Among the tumors, 43 cases were histologically well differentiated (48.8%), whereas 37 (42.1%) were moderately and 8 (9.1) were poorly differentiated. The clinical and pathological parameters of the patients are summarized in **Table 2.**

On univariate analysis to access association of the clinicopathological features with the survival, we obtained that vascular invasion (*p*= 0.02), histological grade (*p*= 0.01), increasing T stage (*p*= 0.03), lymph node metastasis (*p*= 0.01), extracapsular spread of lymph node metastasis (*p*= 0.004) were correlated with overall survival.

All proteins tested were present in the OSCC samples studied and these markers were stained in the membrane of the endothelial cells. **Figure 1** shows representative immunoreactions for D240, CD34 and CD105.

The lymphatic vessels were found more numerously in peritumoral area as well as in areas with inflammatory infiltration. These vessels were thin-walled and clearly distinguished from adjacent blood vessels that were not stained. Occasionally, tumor emboli were observed inside the lymphatic vessels, being more commonly in intratumoral area. In some cases, positive immunostaining for D240 were observed in epithelial cells of the basal layer and tumoral cells.

The blood vessels, stained by CD34 were found in a larger number than the D240, and exhibited a diversity in its morphology. Collapsed, tortuous, small or large were observed both intratumoral and peritumoral. The CD105 has been observed in a less quantity than the other vessels studied and these neoformated vessels were found in intratumoral area with thin-walled appearance, while in peritumoral area its presence were rarely observed.

Eighty-eight cases (100%) exhibited some degree of immunoreactivity to D240 and CD34, thereby indicating the presence of lymphatic vessels and blood vessels, respectively, in the samples analyzed. The average number of lymphatic vessel (LVD) per mm^2 for the 88 cases was 10.6 intratumoral and 10.7 peritumoral. The intratumoral and peritumoral averages blood vessels (BVD) per mm^2 density were 31.1 and 29, while for neoformated vessels (NVD), the values were 8.1 and 3.9.

The association between the immunomarkers and the clinicopathological parameters of the OSCC patients was analyzed. Our results demonstrated that high density of intratumoral LVD was associated with the presence of extracapsular spread of lymph node metastasis (*p*= 0.03), while PT NVD was correlated with pathological nodal status (*p*= 0.05). There was a tendency to statistical significance between the IT LVD and T stage (p= 0.06). No associations were found among BVD with any of the clinicopathologic parameters analyzed (**Table 3**). Recurrence was correlated with IT LVD (*p*< 0.0001), IT BVD (*p*= 0.036) and IT NVD (*p*= 0.047). Overall survival was

associated with the high IT LVD (*p*= 0.0016) and IT NDV (*p*= 0.009) (**Figures 2 and 3**).

In further multivariate analysis based on the Cox proportional hazard model, we found that T stage, histological grade and high IT LVD were independent risk factors for disease-free survival (**Table 4**). Yet, IT LVD was an independent risk factor for overall survival in the same model of analysis (**Table 5**).

DISCUSSION

The TNM classification of oral squamous cell carcinoma is still one of the most important data to categorize the patients and provides a reliable basis for patient prognosis and therapeutic planning (2). Furthermore, more than 50% of patients present advanced stage of the disease and it is strong associated with recurrence and poor survival outcomes (3). In this study, we analyzed the expressions of vascular proteins to assess its densities in patients with advanced stage of the disease (clinical stage III and IV).

Weidner et al (15) first described the correlation between the incidence of metastasis and tumor angiogenesis as measured by microvessel density (MVD) in patients with primary invasive breast carcinoma. This method has also been used for the counting of LVD since then (10).

All cases presented some expression for D240, with averages of 10.6 mm^2 for intratumoral LVD and 10.7 mm^2 for peritumoral LVD. When compared with other studies, there is a significant variation in values of LVD. Values reported in literature include 9 mm^2 from Zhao et al. (17), 33 mm^2 from Franchi (10), 42.9 mm^2 from Maturana-Ramiréz (6) et al*.* and 508 from Bunget et al. (18).

Nodal metastatic involvement is thought to be one of the major survival predictors (3). Our results showed that IT LVD was associated with extracapsular spread of lymph node metastastases (p= 0.03), and this density also tended to significance (p= 0.06) in association with T stage. Some studies of other types of human cancer have indicated a strong correlation between intratumoral and peritumoral LVD and tumor aggressiveness (19, 20). But for OSCC the evidences still controversial (11,12, 17).

This study has found that increasing IT LVD has strong impact in overall survival as well as in recurrence in OSCC patients, in both univariate and multivariate analisys. These findings are in agreement with a study published by Zhao et al. (17) that

observed an increase in the relative risk for poor prognosis in survival outcomes in 5 years.

For microvessel staining, the most common antibodies used are against CD31, CD34 and more recently CD105 (21). Shieh et al. (22) discuss about the number of vessels present in OSCC specimens. They concluded that during the initiation of OSCC the vessels density is more numerously in periphery of the tumor, while as the development of the tumor increasing intratumoral number of vessel is found. Yu (21) and colleagues, in a meta-analysis, concluded that high counts of LVD, but not BVD, seem to be associated with worse prognosis, corroborating with our findings. In 2013, Maturana-Ramiréz et al. (6) published their work where BVD had no relation with any clinicopathological parameter. Similarly, in our work, the correlation of the BVD, intra or peritumoral, revealed no significant differences, based on sex, clinical aspect, histological grade or another parameter used. However, a significant association (p=0.036) with recurrence in 5 years-period was found. Similar result was found in a study performed by Michikawa (23).

Reports on the clinical significance of microvessel density for blood vessels in the head and neck have been contradictory: while some studies found MVD an independent prognostic factor for head and neck SCC (25), others failed to confirm such a correlation (21). This heterogeneity could be explained by the differences in many protocols, in terms of: (i) the origin of the specimens considered (surgically resected material or biopsies, or both), ii) the method used to determine the expressions (conventional pathologic evaluation vs. computer-based image analysis); (iii) quantification methods (vessel count vs. positive area fraction); and (iv) cut-off values Marioni et al. (26). Obviously, these parameters also serve to explain other vessels densities values reported.

CD105 is a trans-membrane glycoprotein expressed on activated vascular endothelial cells composed of two disulphide-linked sub-units of approximately 95 kDa each, forming a 180-kDa homodimeric mature protein (25). This marker has gained attention in recent years and has been used to assess neoformated vessels (9).

A high CD105 expression in primary head and neck squamous cell carcinomas was found associated with cervical lymph node metastases (12, 25, 26) in most of the studies considered. Interestingly, our results have shown the same result in association with peritumoral NVD (p=0.05). In the Schimming and Marme (27) study, CD105 expression was significantly higher in neoplastic tissue than in normal mucosa,

even in peritumoral area. Concerning to our data the presence of vessels stained with CD105 was very rarely in peritumoral tissue.

Martone et al. (28) showed in multivariate analysis that a high microvessel density of CD105+ was the only independent marker of tumor recurrence or death. We found similar results in univariate analysis that demonstrate a significant association between IT NVD and overall survival (p=0.009) and disease-free survival (p=0.047) in 5-year-period.

In conclusion, the results of the current study demonstrate that the assessment of vessels densities in the surgical specimen could provide reliable information in prognosis. Yet, high counts of intratumoral lymphatic density can be considered a useful predictor of aggressiveness in advanced stage OSCC with strong association with survival outcomes. Further studies are required and fundamental investigations and randomized controlled studies with large samples are needed to confirm the prognostic significance of these markers in OSCC patients and their possible clinical application.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by grants of the Conselho Nacional de Desenvolvimento Cientıfico e Tecnologico (CNPq), Brazil.

Ethical Approval

This work was approved by the A.C. Camargo Cancer Center Ethics Committee (Protocol number 1684/12).

REFERENCES

1. Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009; **45**(45):309-16.

2. Curado MP, Johnson NW, Kerr AR, Silva DRME, Lanfranchi H, Pereira DL, Fonseca, FP, Lopes, MA, Kowalski LP, Vargas PA. Oral and oropharynx cancer in South America: Incidence, mortality trends and gaps in public databases as presented to the Global Oral Cancer Forum. Translational Research Oral Oncol 2016; **1**: 1-7.

3. Bettendorf O, Piffko J, Bankfalvi A. Prognostic and predictive factors in oral squamous cell cancer: important tools for planning individual therapy? Oral Oncol 2004; **40**:110-119.

4. Kowalski LP**,** Medina JE. Nodal metastases: predictive factors. Otolaryngol Clin North Am, n.4, 1998; **31**: 621-37

5. Woolgar JA. Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. Oral Oncol 2006; **42**:229–39.

6. Maturana-Ramírez A, Espinoza A, Reyes M, Aitken JP, Aguayo F, Hartel S, Rojas-Alcayaga F. Higher blood vessel density in comparison to the lymphatic vessels in oral squamous cell carcinoma. Int J Clin Exp Pathol. 2015; **8**(10): 13677–686.

7. Folkman J. How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. Cancer Res 1986; **46**: 467-73.

8. Kyzas PA, Agnantis NJ, Stefanou D. Endoglin (CD105) as a prognostic factor in head and neck squamous cell carcinoma. Virchows Arch 2006; **448**: 768–75.

9. Frech S, Hörmann K, Riedel F, Götte K. Lymphatic vessel density in correlation to lymph nodes metastasis in head and neck Squamous Cell Carcinoma. Anticancer Research 2009; **29**: 1675-80.

10. Franchi A, Gallo O, Massi D, Baroni G, Santucci M. Tumor lymphangiogenesis in head and neck squamous cell carcinoma: a morphometric study with clinical correlations. Cancer 2004; **101**: 973-8.

11. Miyahara M, Tanuma J, Sugihara K, Semba I. Tumor lymphangiogenesis correlates with lymph node metastasis and clinicopathologic parameters in oral squamous cell carcinoma. Cancer 2007; **110**: 1287–94.

12. Longatto Filho A, Oliveira TG, Pinheiro C, de Carvalho MB, Curioni OA, Mercante AM, Schmitt FC, Gattás GJ. How useful is the assessment of

lymphatic vascular density in oral carcinoma prognosis? World J Surg Oncol 2007; **5**:140.

13. Sobin LH, Wittekind C. UICC, International Union Against Cancer, TNM classification of malignant tumours. 6th ed. New York: Wiley-Liss; 2002.

14. Wahi PN, Cohen B, Luthra UK, Torloni H Histological typing of oral and oropharyngeal tumours. World Health Organization, Geneva; 1971.

15. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis correlation in invasive breast carcinoma. N Engl J Med 1991; **324**:1–8.

16. Lausen, B. Schumacher, M. Maximally Selected Rank Statistics. Biometrics 1992; **48:** 73–85.

17. Zhao D, Pan J, Li XQ, Wang XY, Tang C, Xuan M. Intratumoral lymphangiogenesis in oral squamous cell carcinoma and its clinicopathological significance. J Oral Pathol Med 2008; **37**: 616-25.

18. Bunget A, Fronie A, Afrem E, Corlan Puscu D, Manolea H, Dan AR, Coman M, Nimigean VR. Microscopic aspects of angiogenesis and lymphangiogenesis in oral squamous cell carcinoma. Rom J Morphol Embryol 2013; **54**: 623- 627

19. Dadras SS, Paul T, Bertoncini J et al. Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. Am J Pathol 2003; **162**: 1951–60.

20. Schoppmann SF, Birner P, Studer P, Breiteneder-Geleff S.Lymphatic microvessel density and lymphovascular invasion assessed by anti-podoplanin immunostaining in human breast cancer. Anticancer Res 2001; **21**: 2351–5.

21. Yu M, Liu L, Liang C, Li P, Ma X, Zhang Q, Wei Y. Intratumoral vessel density as prognostic factors in head and neck squamous cell carcinoma: A meta-analysis of literature. Head Neck 2014; **36**: 596-602.

22. Shieh YS, Lee HS, Shiah SG, Chu YW, Wu CW, Chang LC. Role of angiogenic and non-angiogenic mechanisms in oral squamous cell carcinoma: correlation with histologic differentiation and tumor progression. J Oral Pathol Med 2004; **33**: 601-6.

23. Michikawa C, Uzawa N, Kayamori K, Sonoda I, Ohyama Y, Okada N, Yamaguchi A, Amagasa T. Clinical significance of lymphatic and blood vessel invasion in oral tongue squamous cell carcinomas. Oral Oncol 2012; **48**: 320-4.

24. Chuang HC, Su CY, Huang HY, Chien CY, Chen CM, Huang CC. High expression of CD105 as a prognostic predictor of early tongue cancer. Laryngoscope 2006; **116**: 1175–9.

25. Marioni G, Marino F, Giacomelli L, et al. Endoglin expression is associated with poor oncologic outcome in oral and oropharyngeal carcinoma. Acta Otolaryngol 2006; **126**: 633–9

26. Marioni G, Marino F, Giacomelli L, Staffieri A. CD105 is a marker of tumour vasculature and a potential target for the treatment of head and neck squamous cell carcinoma. J Oral Pathol Med. 2010; **39**(5):361-7.

27. Schimming R, Marme` D. Endoglin (CD105) expression in squamous cell carcinoma of the oral cavity. Head Neck 2002; **24**: 151–6.

28. Martone T, Rosso P, Albera R, et al. Prognostic relevance of CD105 + microvessel density in HNSCC patient outcome. Oral Oncol 2005; **41**: 147–55.

Table 1. Primary serum, clones, source, working titer, and antigen retrieval

Primary Serum	Clone	Source	Working Titer	Antigen Retrieval
D240	D240	DAKO	Ready to use	Citrate pH 6.0
CD105	SN6h	LABVISION	1:50	EDTA pH 9.0
CD34	QBEND10	DAKO	1:400	Citrate pH 6.0

Table 2. Summary of clinicopathological features of OSCC patients

Variables	Categories	Patients	%
Age			
	Range	27 – 82	
	Mean	57	
	Median	58	
Gender			
	Male	64	72.7
	Female	24	27.3
Tobacco Smoking			
	Yes	71	80.6
	No	13	14.7
	n/a	4	4.7
Alcohol consumption			
	Yes	62	70.4
	No	18	20.4
	n/a	8	9.2
Tumor Site			
	Oral tongue	64	72.7
	Floor of the mouth	24	27.3
Tumour Size			
	T1	3	3.4
	T2	17	19.3
	T3	38	43.1
	T4	30	34.2
Nodal Status			
	N0	23	26.1
	N1	28	31.8
	N2	37	42.1
Clinical Stage			
	Stage III	36	40.9
	Stage IV	52	59.1
Histological Grade			
	Well differentiated	43	48.8
	Moderately/poorly differentiated	45	51.2
Recurrence			
	Yes	56	63.7
	No	28	31.8
	n/a	4	4.5
Status			
	Alive	25	28.4
	Dead	61	69.3
	Lost to follow-up	2	2.3

*n/a information not available

Table 3. Association between the vascular densities and clinicopathological characteristics of OSCC patients

Characteristic	Category	No.	Mean Intratumoral LVD (± SD)	P^a	Mean Peritumoral LVD (± SD)	P^a	Mean Intratumoral BVD (± SD)	P^a	Mean Peritumoral BVD (± SD)	P^a	Mean Intratumoral NVD (± SD)	P^a	Mean Peritumoral NVD (± SD)	P^a
Gender	Male	64	11.02 ± 7.15	0.38	9.986 ± 4.81	0.21	30.16 ± 16.1	0.40	29.11 ± 11.2	0.55	8.28 ± 8.23	0.17	4.36 ± 4.37	0.15
	Female	24	9.52 ± 6.99		12.69 ± 4.75		33.43 ± 16.6		30.70 ± 11.5		7.93 ± 8.57		2.96 ± 2.82	
Smoking history	No	11	8.12 ± 8.15	0.19	10.83 ± 3.94	0.98	30.92 ± 15.4	0.76	33.43 ± 9.44	0.11	8.85 ± 10.72	0.82	3.30 ± 1.84	0.84
	Yes	77	10.96 ± 6.92		10.60 ± 5.06		31.97 ± 16.4		28.99 ± 11.4		8.09 ± 11.4		4.07 ± 4.26	
Alcohol	No	26	10.31 ± 6.57	0.79	11.84 ± 4.79	0.16	33.9 ± 18.2	0.31	30.1 ± 10.09	0.71	8.33 ± 9.34	0.92	2.96 ± 2.77	0.06
	Yes	62	10.73 ± 7.36		10.25 ± 4.96		29.8 ± 15.2		29.2 ± 11.8		8.13 ± 7.87		4.40 ± 4.41	
T stage	T1/T2	20	7.94 ± 6.48	0.06	10.76 ± 5.02	0.87	26.74 ± 14.5	0.36	30.45 ± 14.5	0.62	5.16 ± 5.48	0.75	3.56 ± 2.28	0.82
	T3/T4	68	11.39 ± 7.13		10.31 ± 4.92		32.31 ± 16.5		29.28 ± 16.6		9.08 ± 8.77		4.10 ± 4.43	
Pathological Lymph node metastasis	Negative	28	9.81 ± 6.59	0.45	10.99 ± 4.41	0.70	29.9 ± 13.51	0.62	31.5 ± 10.95	0.26	8.92 ± 9.40	0.59	2.95 ± 2.66	**0.05**
	Positive	60	10.98 ± 7.35		10.59 ± 5.16		31.58 ± 17.4		28.6 ± 11.41		7.84 ± 7.76		4.45 ± 4.48	
Histological grade	1*	43	9.93 ± 6.62	0.38	10.6 ± 4.36	0.85	30.4 ± 18.23	0.75	28.7 ± 10.31	0.54	8.18 ± 8.26	0.9	4.49 ± 4.73	0.24
	2**	45	11.2 ± 7.55		10.8 ± 5.44		31.5 ± 14.26		30.2 ± 12.21		8.20 ± 8.39		3.48 ± 3.22	
Vascular invasion	No	53	9.87 ± 6.73	0.24	10.8 ± 4.78	0.73	33.07 ± 15.5	0.16	29.7 ± 11.2	0.83	8.41 ± 8.82	0.74	4.30 ± 4.69	0.30
	Yes	35	11.7 ± 7.59		10.5 ± 5.17		27.99 ± 16.9		29.2 ± 11.4		7.84 ± 7.49		2.90 ± 2.8	
Perineural invasion	No	41	9.85 ± 7.23	0.35	11.38 ± 4.62	0.23	33.8 ± 18.7	0.13	31.05 ± 9.01	0.23	8.22 ± 8.50	0.97	2.90 ± 2.79	0.12
	Yes	47	11.2 ± 6.99		10.14 ± 5.13		26.1 ± 13.3		28.2 ± 12.9		8.16 ± 8.17		4.36 ± 4.83	
Extracapsular spread	No	54	9.32 ± 7.01	**0.03**	10.79 ± 4.94	0.87	28.69 ± 15.8	0.08	30.09 ± 10.8	0.58	8.69 ± 8.80	0.46	3.47 ± 3.08	0.18
	Yes	34	12.6 ± 6.84		10.61 ± 4.95		34.79 ± 16.3		28.68 ± 12.1		7.39 ± 7.43		4.78 ± 5.16	

P^a – p value; * Well differentiated; **Moderately/Poorly differentiated.

Table 4. Multivariate analysis for disease-free survival of advanced stage OSCC patients.

Variables	Categories	*P* value	HR (hazard ratio) multivariate (95% CI)
N stage	0		1.0 (ref)
	1	0.27	1.19 (0.33 – 2.04)
	2	0.015	2.18 (1.24 – 3.84)
Histological Grade	Well-differentiated		1.0 (ref)
	Moderately/poorly differentiated	0.02	1.87 (1.07 – 3.25)
IT LVD	Low	0.001	1.0 (ref)
	High		2.52 (1.38 – 5.59)

Low – When the intratumoral LVD was < 9.93 mm^2

High - When the intratumoral LVD was > 9.93 mm^2

Table 5. Multivariate analysis for global survival of advanced stage OSCC patients.

Variables	Categories	*P* value	HR (hazard ratio) multivariate (95% CI)
T stage	1/2 3/4	0.006	1.0 (ref) 2.29 (1.33 – 5.82)
Histological grade	Well-differentiated Moderately/poorly differentiated	0.004	1.0 (ref) 2.21 (1.28 - 3.81)
IT LVD	Low density High density	0.004	1.0 (ref) 2.19 (1.29 – 3.73)

Low – When the intratumoral LVD was < 9.93 mm^2
High - When the intratumoral LVD was > 9.93 mm^2

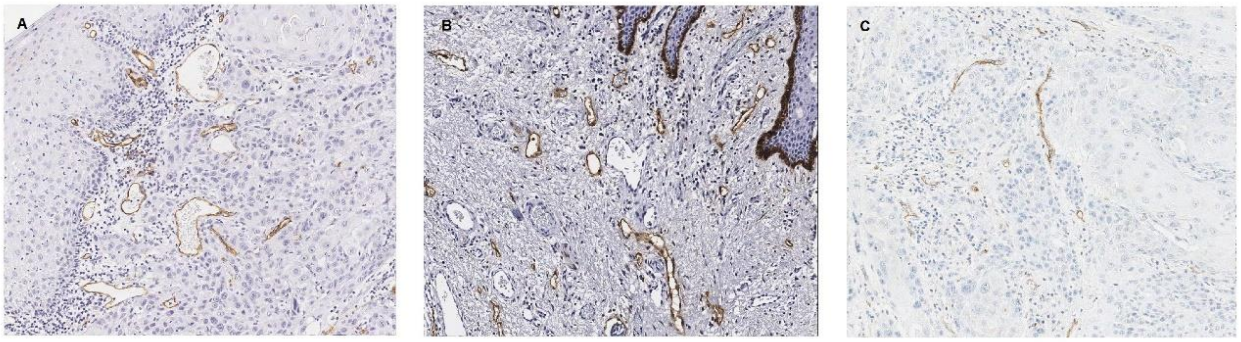

Figure 1. Immunohistochemistry for CD34 (A), D240 (B), and CD105 (C). (magnification x100)

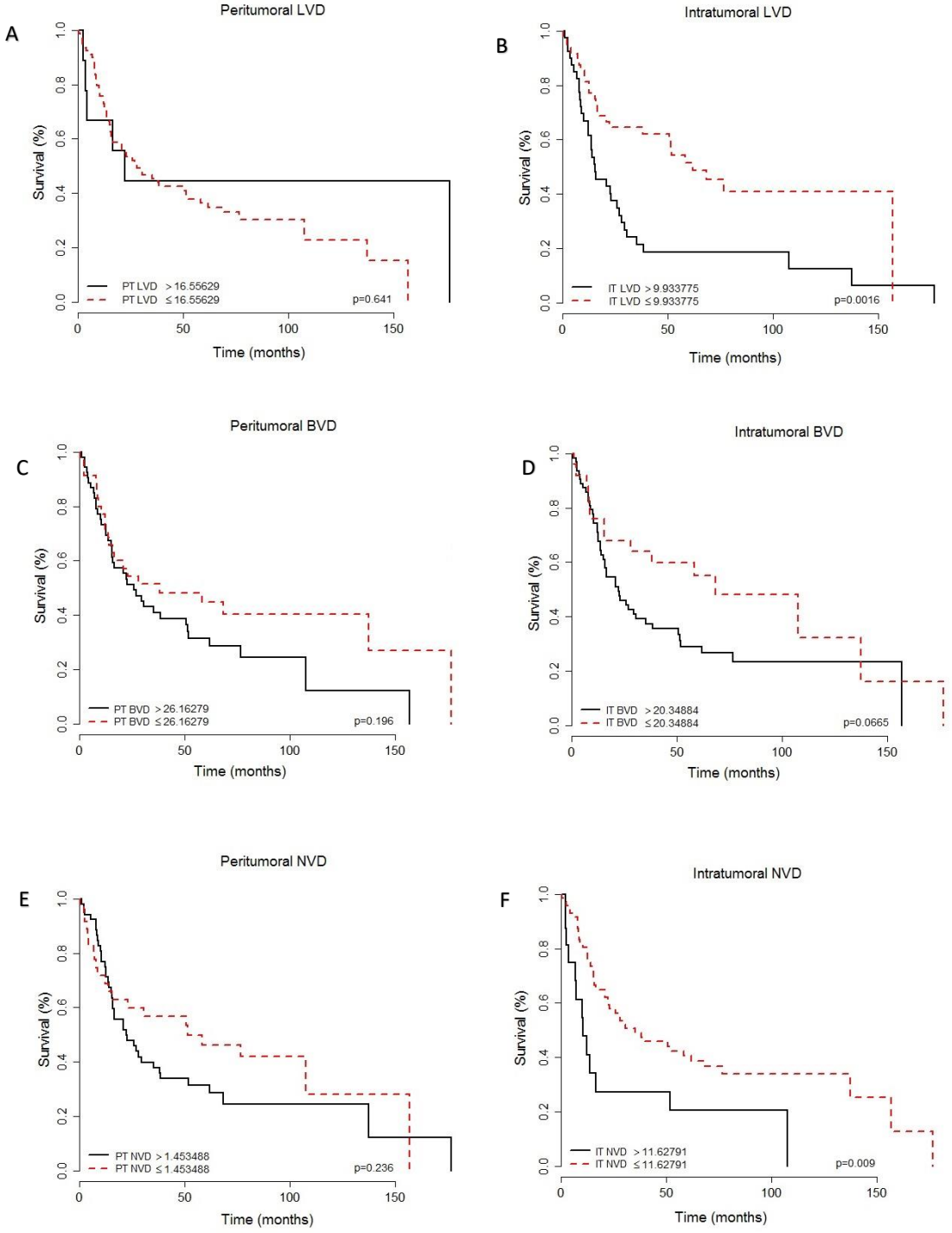

Figure 2. Kaplan-Meier curves for overal survival associated with (A) peritumoral LVD, (B) intratumoral LVD, (C) peritumoral BVD, (D) intratumoral BVD, (E) peritumoral NVD and (F) intratumoral NVD.

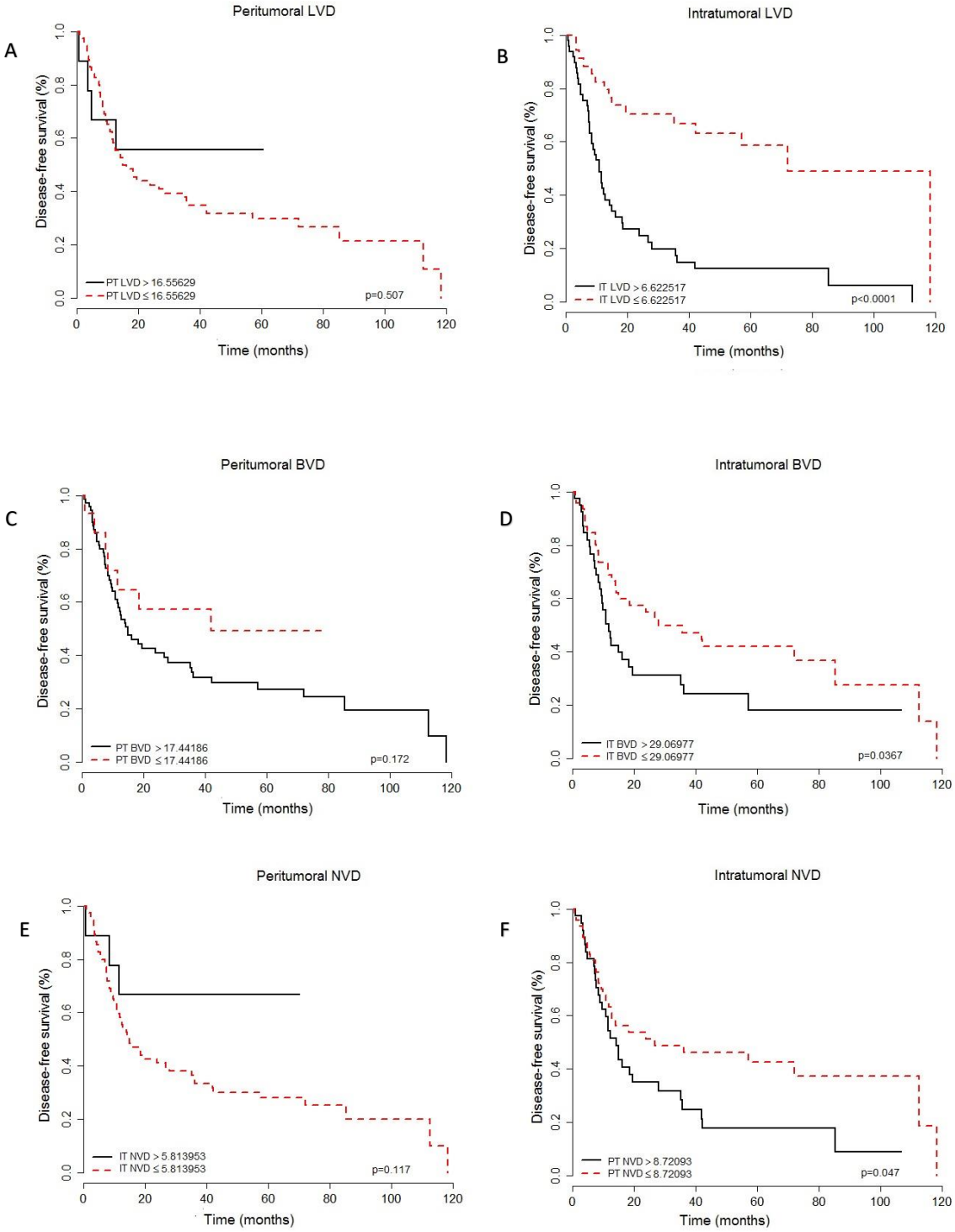

Figure 3. Kaplan-Meier curves for disease-free survival associated with (A) peritumoral LVD, (B) intratumoral LVD, (C) peritumoral BVD, (D) intratumoral BVD, (E) peritumoral NVD and (F) intratumoral NVD.

2.2 Artigo 2: Decreased peritumoral cd1a+ cells predicts worse prognosis in oral squamous cell carcinoma

Artigo em preparação

Juscelino Freitas JARDIM[1], DDS, MSc;
Rogério Gondak[2], DDS, PhD;
Clovis Antonio Lopes PINTO[3], MD, PhD;
Luiz Paulo KOWALSKI[4*], MD, PhD

[1]Department of Oral Diagnosis, Piracicaba Dental School, State University of Campinas
[2]Department of Pathology, Federal University of Santa Catarina
[3]Department of Pathology, AC Camargo Cancer Center
[4]Department of Head and Neck Surgery and Otorhinolaryngology, AC Camargo Cancer Center

Background: Dendritic cells (DCs) are known to play a central role in the regulation of both innate and adaptive immunologic responses, including antitumor immunity. This study aimed to evaluate the prognostic impact of intra and peritumoral dendritic cells in oral squamous cell carcinoma (OSCC) affecting tongue and floor of the mouth. **Patients and Methods:** Immunohistochemical reactions for CD1a and CD83 were performed in 53 patients with OSCC in tongue and floor of the mouth. The markers were evaluated by automated examination in intratumoral and stromal compartments and the results were expressed as the density of cells per mm^2. These data underwent correlation with clinicopathological and survival outcomes. **Results:** Depletion of stromal CD1a+ had association with lymph node metastasis (p = 0.05), whereas the diminished density of stromal CD83+ were correlated with smoking history (p = 0.04), lymph node metastasis (p = 0.015) and extracapsular spread of lymph nodes (p = 0.018). Stromal CD1a+ was correlated with recurrence (p= 0.007) and overall survival (p= 0.03). Results from the survival analysis using the Cox proportional hazard model has shown that decreased number of stromal CD1a+ is an independent factor for overall survival and disease-free survival. **Conclusion:** Our results suggest that the

depletion of number of peritumoral CD1a+ cells is a strong independent prognostic factor, reflecting in higher recurrence rates and lower survival outcomes.
Keywords: Dendritic cells; oral squamous cell carcinoma; oral cancer, Prognosis

Introduction

Oral squamous cell carcinoma (OSCC) accounts for two-thirds of the Head and neck squamous cell carcinoma and is the sixth most common cancer worldwide (1). At the moment of diagnosis more than 50% of all Head and neck squamous cell carcinoma (HNSCC) patients have advanced disease (2), and despite the indication of aggressive and sometimes mutilating therapeutic regimens, overall long term survival is still less than 50%, motivating the search for prognostic factors that can be used to improve our ability to select a more individualized approach according to the risks of recurrence, metastases and death (3).

Oral carcinogenesis is a complex multistep process characterized by several genetic and epigenetic alterations that result in a chaotic cellular growth (3,4). Several biomarkers associated with long term survival results have been identified, and although this growing list of possible biomarkers is extensive, there is currently little transfer of this knowledge into a clinical setting (4).

During the initiation, promotion, and progression of multi-step carcinogenesis, changes in host immunological factors have been observed (5). Dendritic cells (DCs) are a heterogeneous population of cells with high phagocytic activity as immature cells and high cytokine-producing capacity as mature cells that work to maintain a balance between tolerance and protective immunity (6). One of such are the Langerhan's cells (LCs) that are bone marrow-derived dendritic cells (DCs) that represent 2–3% of the entire cell population of normal skin and mucosal epitheliums, and these immature DCs express CD1a (5,7,9), an immune protein responsible for presenting antigens by dendritic Langerhan's cells to T lymphocytes, such as natural killer cells (8, 9). The human CD83 (hCD83) is a 45-kD type-1 membrane glycoprotein member of the Ig superfamily and is one of the best-known maturation markers for human dendritic cells (10).

A relationship between DCs and prognosis in patients affected by different cancers has been previously published (5,11). However, for oral cancer, the present data is still unclear (11). Thus, the purpose of this study was to evaluate the prognostic

impact of intra and peritumoral dendritic cells in OSCC affecting tongue and floor of the mouth.

Patients and Methods

Paraffin-embedded tissue samples from 53 OSCC cases of oral tongue and floor of the mouth were obtained from the Department of Pathology at AC Camargo Cancer Center, São Paulo, Brazil.

All patients were treated at the Department of Head and Neck Surgery and Otorhinolaryngology at the same institution from 2002 to 2010. The eligibility criteria included previously untreated patients, without a second primary tumor and submitted to treatment in the institution. The National Human Research Ethics Committee approved this study (Protocol # 1684/12).

For all cases the medical charts and pathological reports were examined for demographic data (age, gender, tobacco smoking, alcohol consumption, clinical stage and recurrence), clinicopathological information (clinical stage, invasion of adjacent structures, lymphovascular invasion, perineural infiltration, histological grade, pattern of invasion, tumour size, nodal status, and margins).

The tumors were re-staged according to the 2002 version of the International Union Against Cancer (TNM) classification (12). The histological grade was determined on the basis of classification proposed by the World Health Organization (13).

Paraffin-embedded formalin-fixed tissue sections (4 mm) were deparaffinized in xylene and rehydrated in graded ethanol solutions to water. Thereafter, sections were treated with endogenous peroxidase quenching (0.3% H_2O_2 for 15 min) and blocked for unspecific proteins (Novolink Protein Block®, Leica), 20 min each prior to primary antibody incubation. Pressure cooker antigen retrieval consisted of one period at 125°C for 30 min and 90°C for 10 min in EDTA/TRIS (pH 9.0) solution followed by a washing step with phosphate-buffered saline (PBS). Immunohistochemistry using monoclonal antibodies against CD1a (010; Dako, Glostrup, Denmark; 1:400), CD83 (1H4b; Novocastra, Newcastle, UK; 1:200) were used. The incubations with the primary antibodies diluted in PBS were conducted overnight at 4 °C.

The sections were washed and incubated with secondary antibodies (Novolink™ Post Primary, Leica Biosystems, New Castle, UK) for 30 min followed by the polymer detection system (Novolink™ Polymer, Leica Biosystems) for 30 min at

room temperature. Reactions were developed with a solution containing 0.6 mg/ml of 3,30-diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, MO) and 0.01 % H_2O_2 and then counter-stained with Mayer's hematoxylin, dehydrated and mounted with a glass coverslip. Positive controls (a tissue known to contain the antigen under study) were included in all reactions in accordance with manufacturer's protocols.

Immunohistochemical stains were evaluated by automated examination. All slides were digitalized using the Aperio System (Vista, CA, USA), and the resulting images provided were displayed on an LCD monitor with standard contrast, focus, saturation, and white balance. Automated staining intensity was quantified through the Imagescope Software (Aperio System, USA) by the positive pixel count algorithm. The staining intensity was classified as strongly positive (red), positive (orange), weakly positive (yellow) or negative (blue). For counting, we considered valid only the stains with red (strongly positive) and orange (positive). We have selected 5 fields of 1 mm^2 for both, intratumoral and peritumoral, and the results were expressed as the mean positive count staining of the 5 areas analyzed, that were translated into the density of cells per mm^2.

Analysis of the association between protein expressions and the demographic and clinicopathologic characteristics of the patients was performed using the T-student, Mann-Whitney U, Kruska-Wallis and Pearson's chi-square tests as appropriate.

The determination of two group of observations with respect to a simple cut-point was estimate using the maximum of the standardized log-rank statistic proposed by Lausen and Schumacher, 1992 (14). We consider the maximally selected long-rank statistic for cut-points between 5% and 95% quantile of continuous measure.

Overall and disease-free survival probabilities were calculated based on the Kaplan-Meier estimator. The comparison among survival functions were assessed through of logrank test. Relative risk was evaluated by the Cox proportional hazards model. Statistical analyses were performed using using SPSS (version 23) and R software version 3.2.1 and the significance level was fixed at 5% for all statistical tests.

RESULTS

Among 53 eligible patients, 40 (75.5%) were male, and 13 (25.5%) were female. Most of the patients reported smoking (42 patients, 79.2%) and alcohol drinking (36 patients, 67.9%). The patients' mean age was 56 years. In 67.9% (36 cases) of cases

the oral tongue was the primary site, while in 32.1% (17 cases) the tumor arised in the floor of the mouth. Twenty-five (47.2%) patients had their tumors classified as histological well differentiated, whereas 28 (52.8%) were classified as moderately/poorly differentiated.

Only 26 cases (49.1%) were node negative and among the cases with positive nodes, 13 (24.5%) were N1 and 14 (26.4%) were N2. Most of the cases (43.4%) were TNM stage III. The clinicopathological data are summarized in **Table 1**.

On univariate analysis to access association of the clinicopathological features with the survival, histological grade (p= 0.027), increasing T stage (p= 0.001), lymph node metastasis (p= 0.001), clinical stage (p= 0.01) and perineural invasion were correlated with overall survival. Disease-free survival rates were influenced by perineural invasion (p=0.01), T stage (p=0.028), N stage (p=0.024), clinical stage (p=0.03) and histological grade (p=0.038).

Both (CD1a and CD83) proteins tested were present in the OSCC samples studied and these markers were stained in the membrane of the DCs. These cells were identified as ramified cell in neoplastic epithelium and connective tissue. Examples of immunostaining for CD1a and CD83 are shown in **Figure 1**. The average number for the 53 cases of DCs stained by CD1a was 20.65/mm^2 intratumoral and 10.24 /mm^2 in stromal. For CD83 the values were 7.74/mm^2 and 9.75/mm^2, respectively.

The association between the immunomarkers and the clinicopathological parameters of the OSCC patients was analyzed (**Table 2**). It was shown that a depletion number of stromal CD1a+ had association with lymph node metastasis (p = 0.05) and a tendency to significance (p = 0.09) with extracapsular spread of lymph node metastases. Diminished counts of stromal CD83+ were correlated with smoking history (p = 0.04), lymph node metastasis (p = 0.015) and extracapsular spread (p = 0.018). Correlation analysis of intratumoral CD1a+ counts did not reveal any statistically significance finding, whereas intratumoral CD83+ tended to be associated with histological grade (p = 0.089). Stromal CD1a+ was correlated with Recurrence (p= 0.007) and overall survival rates (p= 0.03) (**Figures 2 and 3**).

The multivariate survival analysis using the Cox proportional hazard model showed that T stage, extracapsular spread of lymph nodes and decrease number of CD1a+ were independent factors associated with disease-free survival (**Table 3**). In addition, CD1a+ was also an independent risk factor for overall survival in the same model of analysis (**Table 4**).

DISCUSSION

The immune system is able to detect and eliminate emerging malignant cells to prevent their uncontrolled proliferation. This process of cancer immune surveillance is an important host protection process to inhibit carcinogenesis and to maintain cellular homeostasis (15). In this context, Dendritic cells are known to play a central role in the regulation of both innate and adaptive immunologic responses, including antitumor immunity (7).

The function of DCs is to recognize antigen, process it and present it to T cells, including recognition of tumor molecules, capture and cross-presentation of released-tumor-associated antigens (6, 16). Moreover, these cells have been described to be capable of inducing tumor cell death by mechanisms such as fas or nitric oxide mediated interactions (16, 17).

There are, currently, a large number of markers targeting DCs that are able to recognize proteins like S100, Cd1a, CD83, CD207, CD208, CD80, CD11c, CD86 and HLA-DR (18). In this study, we have selected the CD1a and CD83, because the well-established biological properties and previous investigations in different neoplasms. It is accepted that CD1a is a marker of immature DCs, such as Langerhans cells, whereas CD83 is one of the well-known maturation markers for human DC and has been shown to be expressed on activated and mature cells (19).

Infiltration of tumors by DCs reflects the host immune defense mechanism and has been associated with better prognosis, reduced tumor recurrence rates, and fewer metastasis (17, 19, 20). This condition was shown in some malignancies, such as breast (21), gastric (22), pancreatic (23) and laryngeal (24) cancers. Therefore, it is possible to suppose that the participation of dendritic cells in antitumor immunity indicates that the behavior of these cells can be directly related to disease progression (25, 26).

Gomes et al. (18) has shown a decreased number of CD1a+ in lower lip squamous cells carcinoma when compared to normal epithelium. Their results suggested that the reduced counts of LCs in epithelium would represent an important step for lip cancer development. It is believed that inadequate presentation of antigens by the host Langerhans cells is one potential mechanism that allows tumor progression (11).

Several researchers have been investigating the distribution of Langerhans cells in HNSCC, however the currently data is controversial (17, 27). While some authors

(28) conclude an increased number of LC, others noticed a reduced number of Langerhans cells (29, 30). However, most investigators commonly concluded that there was a significant decrease of these cells in tumors when compared with non-malignant or surrounding tissues (17,18, 27).

Two studies of laryngeal squamous cell carcinomas demonstrated that high counts of DCs infiltration was found to be associated with longer disease-free survival (20, 24). On the other hand, Rodríguez Sanjuán et al. did not find any correlation with survival outcomes in esophageal carcinomas (31).

Nodal metastasis is a critical step in the progression of malignant tumors and is one of the most important prognostic factors in OSCC. In our study, we report that fewer stromal CD1a+ and CD83+ cells had association with a higher risk of lymph node metastasis. This data is in accordance with Goldman et al. that observed a significant decreased CD1a+ cells number in association with lymph node metastasis (32).

Many authors have been observed that LCs were more abundant in well/moderately differentiated than in poorly differentiated OSCC (11,29). In contrast, we did not reach this result, corroborating with a work published by van Herden et al (33). However, we found a tendency to statistical significance between the diminished counts of intratumoral CD83+ with histological grade ($p=0.08$).

In this work, we found that stromal depletion of CD1a+ was an independent factor significantly associated with overall survival and risk of recurrence. Similar findings were shown in a study performed by Kindt et al. (30) that demonstrated that the number of LCs is a strong and independent prognostic marker for HNSCC patients in terms of recurrence when these number is evaluated in the intratumoral and stromal compartments and in terms of overall survival when analyzed in the stromal compartment. Goldman et al. (32) have concluded that greater number of CD1+ cells adjacent to the tongue carcinoma exhibited a better prognosis with reduced recurrence rate and improved survival compared with those with lower number. Yet, in the same study, the authors stated that CD1a-positive peritumoral subpopulation of DCs is functionally distinct and is more important to antitumor immunity than the other subpopulations.

Dendritic cells are potentially good candidate for use in therapeutic cancer vaccines. The aim of DC vaccination is to induce tumour-specific effector T cells that can reduce the tumour mass specifically and that can induce immunological memory to reduce the risk of cancer recurrence (34, 35).

In conclusion, our results suggest that the depletion of number of peritumoral CD1a+ cells is a strong independent prognostic factor, reflecting in higher recurrence rates and lower survival outcomes. Therefore, the distribution of DCs in OSCC tumor tissues could be considered as a good prognosticator tool.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by grants of the Conselho Nacional de Desenvolvimento Cientıfico e Tecnologico (CNPq), Brazil.

Ethical Approval

This work was approved by the A.C. Camargo Cancer Center Ethics Committee (Protocol number 1684/12).

REFERENCES

1. Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009; **45**(45):309-16.

2. Curado MP, Johnson NW, Kerr AR, Silva DRME, Lanfranchi H, Pereira DL, Fonseca, FP, Lopes, MA, Kowalski LP, Vargas PA. Oral and oropharynx cancer in South America: Incidence, mortality trends and gaps in public databases as presented to the Global Oral Cancer Forum. Translational Research Oral Oncol 2016; **1**: 1-7.

3. Bettendorf O, Piffko J, Bankfalvi A. Prognostic and predictive factors in oral squamous cell cancer: important tools for planning individual therapy? Oral Oncol 2004; **40**:110-119.

4. Sinevici N, O'sullivan J. Oral cancer: Deregulated molecular events and their use as biomarkers. Oral Oncol 2016; **61**: 12–18.

5. Bennaceur K, Chapman J, Brikci-Nigassa L, Sanhadji K, Touraine JL, Portoukalian J. Dendritic cells dysfunction in tumor environment. Cancer Lett 2008; **272**:186-96.

6. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; **392**; 245–252.

7. Austyn JM. The dendritic cell system and anti-tumor immunity. In Vivo 1993; **7**:193-201

8. Coventry B, Heinzel S. CD1a in human cancers: A new role for an old molecule. Trends Immunol 2004**; 25**:242-8.

9. Gondak RO, Alves DB, Silva LF, Mauad T, Vargas PA. Depletion of Langerhans cells in the tongue from patients with advanced-stage acquired immune deficiency syndrome: relation to opportunistic infections. Histopathology 2012; 60: 497–503.

10. Bachereau J, Briere F, Caux C, *et al.* Immunobiology of dendritic cells. Ann Rev Immunol, 2000, **18**: 767–811.

11. Upadhyay J, Rao NN, Upadhyay RB. A comparative analysis of langerhans cell in oral epithelial dysplasia and oral squamous cell carcinoma using antibody CD-1a. J Can Res Ther 2012; **8**: 591-7.

12. Sobin LH, Wittekind C. UICC, International Union Against Cancer, TNM classification of malignant tumours. 6th ed. New York: Wiley-Liss; 2002.

13. Wahi PN, Cohen B, Luthra UK, Torloni H Histological typing of oral and oropharyngeal tumours. World Health Organization, Geneva; 1971.

14. Lausen, B. Schumacher, M. Maximally Selected Rank Statistics. Biometrics 1992; **48:** 73–85.

15. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology 2007;**121**(1):1-14.

16. Huang J, Tatsumi T, Pizzoferrato T, et al. Nitric oxide sensitizes tumor cells to dendritic cell-mediated apoptosis, uptake, and cross-presentation. Cancer Res 2005; **6**: 8461-70.

17. Upadhyay RB, Upadhyay J, Rao NN, Agrawal P. The role of Langerhans cells in oral squamous cell carcinoma. Chin Ger J Clin Oncol 2011; **10**: 606-11.

18. Gomes JO, Carvalho MV, Fonseca FP, Gondak RO, Lopes MA, Vargas PA. CD1a+ and CD83+ Langerhans cells are reduced in lower lip squamous cell carcinoma. J Oral Pathol Med 2016; **45**: 433–439.

19. Perez L, Shurin MR, Collins B, Kogan D, Tourkova IL, Shurin GV. Comparative analysis of CD1a, S-100, CD83, and CD11c human dendritic cells in normal, premalignant, and malignant tissues. Histol Histopathol 2005**; 20**: 1165–72.

20. Gallo O, Libonati GA, Gallina E, et al. Langerhans cells related to prognosis in patients with laryngeal carcinoma. Arch Otolaryngol Head Neck Surg 1991; 117:1007–10.

21. Iwamoto M, Shinohara H, Miyamoto A, et al. Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. Int J Cancer 2003; 104: 92–7.

22. Hu M, Li K, Maskey N, et al. Decreased intratumoral Foxp3 Tregs and increased dendritic cell density by neoadjuvant chemotherapy associated with favorable prognosis in advanced gastric cancer. Int J Clin Exp Pathol 2014; 7: 4685–94.

23. Dallal RM, Christakos P, Lee K, et al. Paucity of dendritic cells in pancreatic cancer. Surgery 2002; 131: 135–8.

24. Yilmaz T, Gedikoglu G, Celik A, Onerci M, Turan E. Prognostic significance of Langerhans cell infiltration in cancer of the larynx. Otolaryngol Head Neck Surg. 2005 ;132(2):309-16.

25. De Ávila ED, Molon RS, Lawall MA, Consolaro RB, Consolaro A. Increased of Langerhans Cells in Smokeless Tobacco-Associated Oral Mucosal Lesions. Arch Clin Exp Surg 2012; 1: 85-93.

26. Wright-Browne V, McClain KL, Talpaz M, Ordonez N, Estrov Z. Physiology and pathophysiology of dendritic cells. Hum Pathol 1997; 28:563-579.
27. Lasisi TJ, Oluwasola AO, Lasisi OA, Akang EE. Association between langerhans cells population and histological grade of oral squamous cell carcinoma. J Oral Maxillofac Pathol 2013;**17**(3):329-333.

28. Rani SV, Aravindha B, Leena S, Balachander N, Malathi LK, Masthan MK. Role of abnormal Langerhans cells in oral epithelial dysplasia and oral squamous cell carcinoma: A pilot study. J Nat Sci Biol Med. 2015;6(Suppl 1): S128-S133.

29. Kurihara K, Hashimoto N. The pathologic significance of Langerhans cell in oral cancer. J Oral Pathol 1985; 14:289-98.

30. Kindt N, Descamps G, Seminerio I, et al. Langerhans cell number is a strong and independent prognostic factor for head and neck squamous cell carcinomas. Oral Oncol 2016; **62**:1-10.

31. Rodríguez Sanjuán JC, Val-Bernal F, Buelta CL, et al. Langerhans cells do not influence the postoperative survival of esophageal epidermoid carcinoma. Rev Esp Enferm Dig, 1993, 84: 143–147.

32. Goldman SA, Baker E, Weyant RJ, Clarke MR, Myers JN, Lotze MT. Peritumoral CD1a positive dendritic cells are associated with improved survival in patients with tongue carcinoma. Arch Otolaryngol Head Neck Surg 1998;124:641-6.

33. Van Heerden WFP, Raubenheimer EJ, van Rensburg EJ, le Roux R. Lack of correlation between DNA ploidy, Langerhans cell population and grading in oral squamous cell carcinoma. J Oral Pathol Med 1995; 24: 61–5.

34. Palucka K, Ueno H, Fay J, Banchereau J. Dendritic cells and immunity against cancer. J Intern Med 2011; **269**:64–73.

35. Zhang W, Yu X, Kwak M, et al. Maturation of dendritic cells by pullulan promotes anti-cancer effect. Oncotarget 2016; **7**(28):44644-44659.

Table 1. Summary of clinicopathological features of OSCC patients

Variables	Categories	Patients	%
Age			
	Range	27 – 82	
	Mean	56	
	Median	59	
Gender			
	Male	40	75.5
	Female	13	25.5
Tobacco Smoking			
	Yes	42	79.2
	No	11	20.8
Alcohol consumption			
	Yes	36	67.9
	No	17	32.1
Tumor Site			
	Tongue	37	69.8
	Floor of the mouth	16	30.2
Tumour Size			
	T1	6	11.3
	T2	15	28.3
	T3	20	37.7
	T4	12	22.6
Nodal Status			
	N0	26	49.1
	N1	13	24.5
	N2	14	26.4
Clinical Stage			
	Stage I	7	13.2
	Stage II	7	13.2
	Stage III	23	43.4
	Stage IV	16	30.2
Histological Grade			
	Well differentiated	25	47.2
	Moderately/poorly differentiated	28	52.8
Recurrence			
	Yes	33	62.3
	No	19	35.7
	n/a	1	1.9
Status			
	Alive	17	32.1
	Dead	34	64.2
	Lost to follow-up	2	3.8

*n/a information not available

Table 2 . Association between the CD1a+ and CD83+densities and clinicopathological characteristics of OSCC patients

Characteristic	Category	No.	Mean Intratumoral CD1a (± SD)	P^a	Mean Stromal CD1a (± SD)	P^a	Mean Intratumoral CD83 (± SD)	P^a	Mean Stromal CD83 (± SD)	P^a
Gender	Male	40	20.48 ± 12.6	0.73	10 ± 6.15	0.9	7.24 ± 6.48	0.69	8.90 ± 5.76	0.11
	Female	13	21.15 ± 11.3		10.98 ± 8.43		9.26 ± 8.75		12.36 ± 7.11	
Smoking history	No	11	21.06 ± 9.59	0.53	11.6 ± 9.33	0.70	8.45 ± 6.39	0.6	12.65 ± 6.11	**0.04**
	Yes	42	20.5 ± 12.9		9.86 ± 5.92		7.55 ± 7.30		8.99 ± 6.10	
Alcohol	No	17	22.1 ± 11.9	0.4	11.6 ± 7.45	0.35	8.97 ± 7.75	0.51	11.35 ± 7.24	0.23
	Yes	36	19.9 ± 12.4		9.56 ± 6.32		7.15 ± 6.76		9 ± 5.64	
T stage	T1/T2	21	21.11 ± 11.2	0.9	11.13 ± 7.54	0.6	8.45 ± 7.61	0.64	10.7 ± 6.62	0.82
	T3/T4	32	20.34 ± 12.9		9.65 ± 6.15		7.27 ± 6.78		9.13 ± 5.98	
Lymph node metastasis	Negative	17	21.17 ± 12.2	0.54	12 ± 6.6	**0.05**	8.72 ± 7,31	0.14	11.13 ± 6.31	**0.015**
	Positive	36	19.54 ± 12.5		6.50 ± 5.22		5.66 ± 6.22		6.83 ± 5.07	
Histological grade	1*	25	19.9 ± 10.5	0.97	10.5 ± 6.76	0.76	9.10 ± 6.54	0.08	10.27 ± 5.75	0.45
	2**	28	21.3 ± 13.7		9.99 ± 6.78		6.52 ± 7.42		9.29 ± 6.69	
Vascular invasion	No	32	20.4 ± 10.6	0.7	9.38 ± 6.97	0.15	6.57 ± 6.84	0.13	8.87 ± 6.09	0.17
	Yes	21	20.9 ± 14.6		11.5 ± 6.21		9.52 ± 7.20		11.09 ± 6.33	
Perineural invasion	No	23	23.5 ± 13.5	0.16	10.5 ± 7.33	0.9	8.88 ± 7.76	0.44	11.2 ± 7.13	0.29
	Yes	30	18.4 ± 10.7		9.97 ± 6.3		6.86 ± 6.49		8.62 ± 5.29	
Extracapsular spread	No	33	19.9 ± 12	0.58	12.1 ± 6.98	0.09	8.51 ± 7.21	0.47	11.9 ± 6.09	**0.018**
	Yes	20	21.8 ± 12.8		9.09 ± 6.36		7.27 ± 7.06		8.41 ± 6.01	

Man-Whitney U Test; P^a – *p* value; * - Well-differentiated ** - Moderately /poorly differentiated

Table 3. Multivariate analysis for disease-free survival of OSCC patients.

Variables	Categories	*P* value	HR (hazard ratio) multivariate (95% CI)
T stage	1/2	0.006	1.0 (ref)
	3/4		3.67 (1.45. – 9.24)
Extracapsular spread	pN -		1.0 (ref)
	pN +CR-	0.09	2.01 (0.75 - 5.37)
	pN +CR+	0.03	2.84 (1.08 – 7.42)
Stromal CD1a	> 8 cells / mm^2	0.001	1.0 (ref)
	< 8 cells / mm^2		4.23 (1.96 – 9.17)

pN – Negative lymph node; pN+CR- Positive lymph node with no extranodal spread; pN+CR+ Positive lymph node with extracapsular spread

Table 4. Multivariate analysis for global survival of OSCC patients.

Variables	Categories	*P* value	HR (hazard ratio) multivariate (95% CI)
T stage	1/2	0.001	1.0 (ref)
	3/4		5.37 (2.06 –13. 97)
Extracapsular spread	pN -		1.0 (ref)
	pN +CR-	0.05	1.59 (0.6 - 4.7)
	pN +CR+	0.01	3.24 (1.23 – 8.52)
Histological grade	Well-differentiated		1.0 (ref)
	Moderately/poorly differentiated	0.002	3.57 (1.61 – 7.90)
Stromal CD1a	> 8 cells / mm^2	0.001	1.0 (ref)
	< 8 cells / mm^2		3.61 (1.63 – 7.96)

pN – Negative lymph node; pN+CR- Positive lymph node with no extranodal spread; pN+CR+ Positive lymph node with extracapsular spread

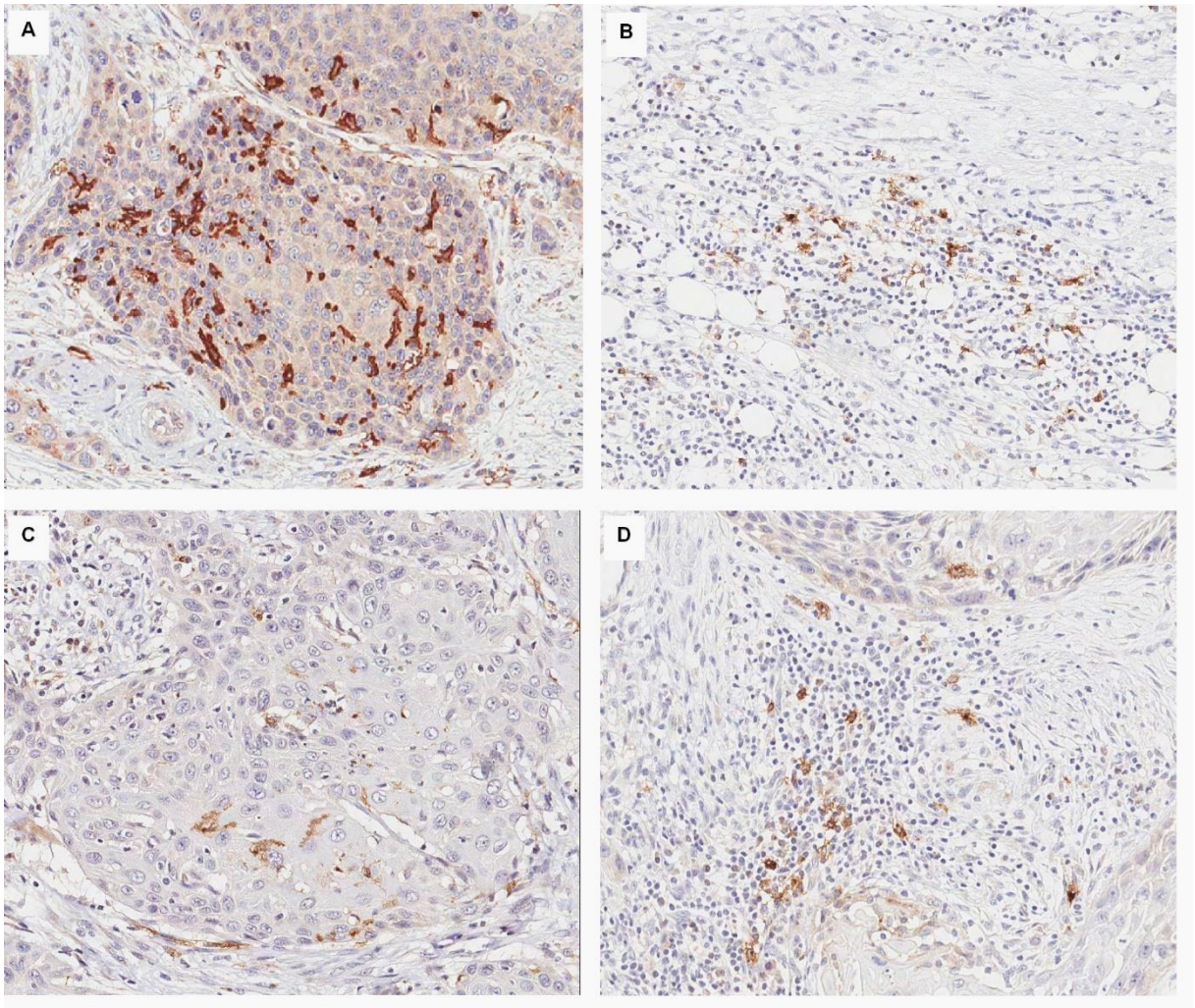

Figure 1. Immunohistochemical detection of dendritic cells with CD1a+ in (A) intratumoral (200x) area and (B) in stromal tissue (100x). Imunnohistochemical expression of CD83+ in (C) intratumoral (200x) and (D) stromal (100x) fields.

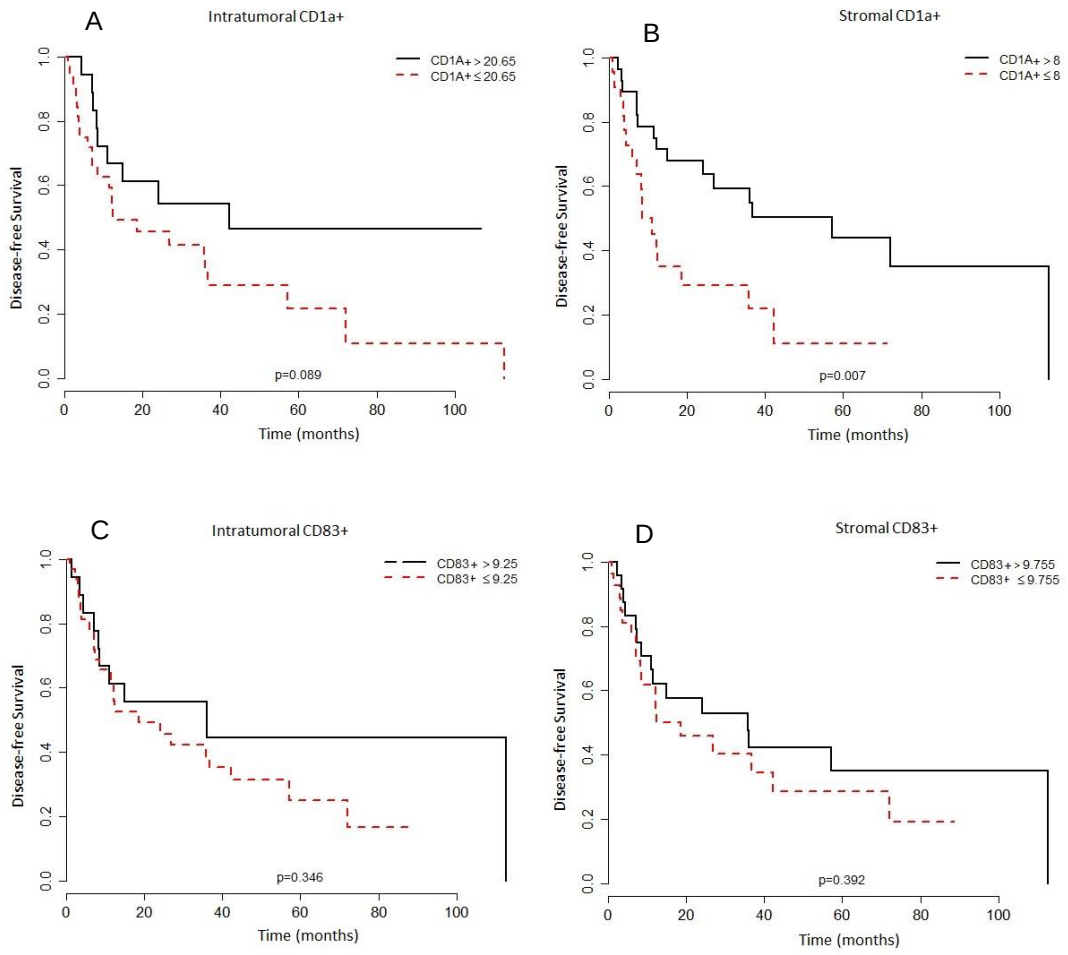

Figure 2. Kaplan-Meier curves for disease-free survival associated with (A) intratumoral CD1a+, (B) stromal CD1a+, (C) intratumoral CD83+, (D) stromal CD83+.

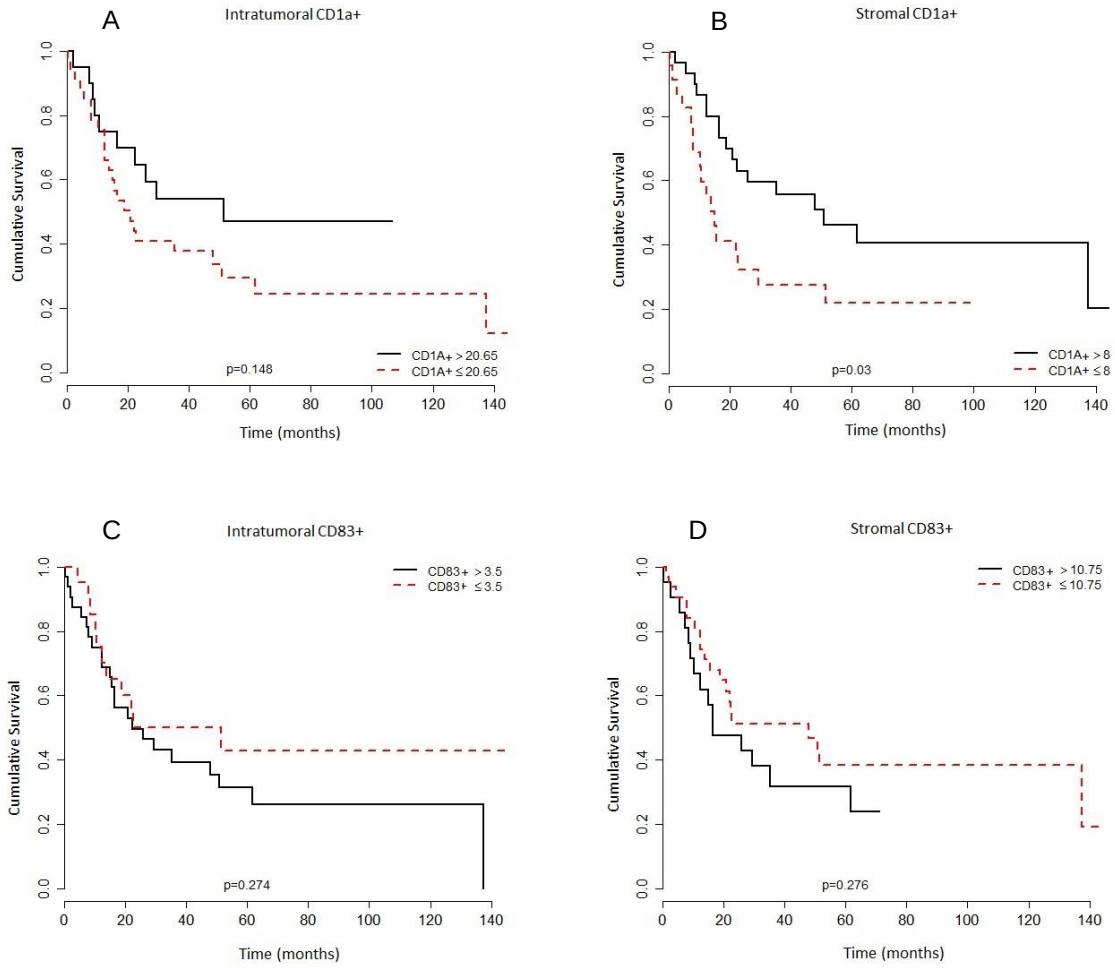

Figure 3. Kaplan-Meier curves for overall survival associated with (A) intratumoral CD1a+, (B) stromal CD1a+, (C) intratumoral CD83+, (D) stromal CD83+.

3 DISCUSSÃO

O sistema TNM para o carcinoma epidermóide de cavidade bucal fornece uma base confiável para o prognóstico do paciente e o planejamento terapêutico (Curado et al., 2012). Infelizmente, mais de 50% dos pacientes apresentam estágio avançado da doença no momento do diagnóstico, e, portanto, apresentam altas taxas de recidiva e pobre expectativa de sobrevida em 5 anos (Warnakulasuriya et al., 2009).

Tumores malignos também têm a habilidade de induzir o crescimento de novos vasos sanguíneos a partir de vasos periféricos (angiogênese), que são importantes para a progressão tumoral, crescimento, agressividade e habilidade para produzir metástases (Folkman, 1995; Massano et al., 2006). Segundo Schimming et al. (2004) e Nagatsuka et al. (2005), a endoglina (CD105) é o marcador endotelial com melhor especificidade para avaliação do padrão da angiogênese em carcinomas de células escamosas bucais, pois esta glicoproteina está envolvida no processo de indução da neovascularização.

Segundo Marione et al (2006) significado clínico da microdensidade vascular em câncer de cabeça e pescoço têm sido contraditórios: enquanto alguns estudos encontraram MVD um fator prognóstico independente para CEC de cabeça e pescoço, outros não confirmaram essa correlação (Yu et., 2014). Marioni et al. (2010) afirmam que essa heterogeneidade pode ser explicada pelas diferenças em muitos protocolos, em termos de: (i) a origem dos espécimes considerados (material proveniente de biópsias, ressecções ou ambos), ii) o método utilizado para determinar as expressões (avaliação patológica convencional ou análise de imagens por computador); (iii) métodos de quantificação (contagem de vasos ou porcentagem de área positiva); e (iv) valores de *cutoff*.

Weidner et al (1991) descreveram pela primeira vez a correlação entre a incidência de metástase e angiogênese tumoral, medida pela densidade de microvasos (DMV) em pacientes com carcinoma mamário invasivo primário. Este método também tem sido utilizado para a contagem de vasos linfáticos desde então.

Em nosso trabalho constatamos que o aumento da densidade intratumoral de vasos linfáticos teve um forte impacto na sobrevida global, bem como na recorrência em pacientes com CEC, tanto em análises univariadas como multivariadas. Esses achados estão de acordo com um estudo publicado por Zhao et

al. (2008) que observou um aumento no risco relativo de pior prognóstico nos resultados de sobrevida em 5 anos.

Acredita-se que o processo de linfangiogênese (formação de novos vasos linfáticos) seja crucial para que as células cancerosas migrem para os linfonodos regionais. A densidade linfática, aferida pela imunomarcação de D2-40, foi significativamente correlacionada a metástase linfonodal ($p<0,001$) em carcinoma epidermóide de boca no estudo de Sugiura et al. (2009) e foi um fator de risco independente para metástase linfonodal em carcinomas de língua no estudo de Zhang et al. (2011). Em concordância com estes estudos, evidenciamos que a densidade de vasos linfáticos intratumorais tinha relação com a ruptura de cápsulas linfonodais acometidos por metástases.

Uma alta expressão de CD105 em carcinomas de células escamosas de cabeça e pescoço foi encontrada associada a metástases linfonodais (12, 25, 26) na maioria dos estudos considerados. Curiosamente, nossos resultados mostraram o mesmo resultado em associação com densidade de vasos neoformados na região peritumoral ($p = 0,05$). No estudo de Schimming e Marme (2002), a expressão de CD105 foi significativamente maior no tecido neoplásico do que na mucosa normal, mesmo na região peritumoral. Em nossa casuística, raramente observamos a presença de vasos CD105+ no tecido peritumoral.

Martone et al. (2005) mostraram na análise multivariada que uma alta densidade de microvasos CD105 + foi o único marcador independente de recorrência tumoral e sobrevida. Nosso trabalho evidenciou resultados semelhantes na análise univariada, a cuja associação significativa entre a densidade de neovasos intratumorais e a sobrevida global ($p = 0,009$) e a sobrevida livre de doença ($p = 0,047$) em um período de 5 anos, foram observadas.

Por sua vez, sistema imunológico é capaz de detectar e eliminar as células malignas emergentes para evitar a sua proliferação descontrolada. Este processo de vigilância imunológica do câncer é um importante processo de proteção do hospedeiro para inibir a carcinogênese e manter a homeostase celular (Kim et al.,2007) . Neste contexto, sabe-se que as células dendríticas (CDs) desempenham um papel central na regulação de respostas imunológicas inatas e adaptativas, incluindo a imunidade antitumoral (Austin, 1993).

A função das CDs é reconhecer o antígeno, processá-lo e apresentá-lo às células T, incluindo reconhecimento de moléculas tumorais. Atualmente um grande número de marcadores direcionados para células dendríticas capazes de reconhecer proteínas como S100, Cd1a, CD83, CD207, CD208, CD80, CD11c, CD86 estão disponíveis (Gomes et al., 2016). Neste estudo, selecionamos o CD1a e o CD83, por conta das de suas propriedades biológicas bem conhecidas e investigações prévias em diferentes neoplasias. É aceito que CD1a é um marcador de células dendríticas imaturas, tais como células de Langerhans, enquanto que CD83 é um dos marcadores de maturação mais bem reconhecidos para a célula dendrítica humana e demonstrou ser expresso em células ativadas e maduras (Perez et a., 2005).

A infiltração de CDs em tumores reflete o mecanismo de defesa imune do hospedeiro e tem sido associada com melhor prognóstico, menor taxa de recorrência de tumores e menores índices de metástases (Upadhyay et al., 2011). Isto foi mostrado em algumas neoplasias malignas, tais como cancro da mama (Iwamoto et al., 2003), gástrico (Hu et al., 2014) e laríngeo (Yilmaz et al., 2005). Portanto, é possível supor que a participação de células dendríticas na imunidade antitumoral indica que o comportamento dessas células pode estar diretamente relacionado à progressão da doença (Wright-Browne et a., 1997).

Gomes et al. (2016) mostrou um número diminuído de CD1a + no carcinoma de células escamosas quando comparado ao epitélio normal. Seus resultados sugerem que a redução da contagem de células de Langherans no epitélio representaria um passo importante para o desenvolvimento do câncer labial. Acredita-se que a apresentação inadequada de antígenos pelo hospedeiro pelas CDs é um potencial mecanismo que permite a progressão do tumor (11).

A relação entre células dendríticas e prognóstico tem sido relatada em malignidades humanas, contudo a relação com o CEC de boca permanece bastante dúbia. Em nosso estudo identificamos que a redução de células CD1a+ localizadas na região peritumoral, tinha relação direta com o prognóstico em CECs de língua e assoalho de boca. Corroborando com nossos resultados Kindt et al. (2016) que demonstraram que o número de células de Langherans é um forte marcador prognóstico para pacientes com câncer de cabeça e pescoço em termos de recorrência quando em associação com as regiões peritumoral e intratumoral, e em termos de sobrevida global quando em correção com baixas contagens de CDs na

região peritumoral. Em outro estudo, Goldman et al. (1998) evidenciaram que pacientes com CEC de boca, apresentando alta expressão CD1a+, apresentaram um melhor prognóstico, impactando na sobrevida global e reduzindo taxas de recidiva, corroborando com nosso estudo.

4 CONCLUSÃO

Em conclusão, o CEC de língua e assoalho bucal acomete principalmente homens acima de 55 anos. Fatores como tabaco e bebidas alcólicas estão fortemente associadas à doença. O espécime cirúrgico histopatológico fornece importantes indícios prognósticos, dentre os quais a invasão perineural e o grau histológico podem ser citados. Evidenciou-se que a densidade vascular linfática intratumoral, expressada pela imunomarcação de D240, é um bom preditor de sobrevida, uma vez que altas contagens de sua expressão estão relacionadas com recorrência tumoral e pobres taxas de sobrevivência. Vasos neoformados, marcados por CD105, em contagens elevadas no tecido peritumoral tem uma relação com metástases linfonodais. O aumento da densidade global de vasos sanguíneos, expressadas pelo marcador CD34 exibiu relação com recorrência. Ainda, no que concerne ao sistema imune, a depleção de células dendríticas, principalmente, as presentes no tecido áre de estroma tumoral, exibem uma correlação com baixas taxas de sobrevida global e com recorrência da doença.

REFERÊNCIAS

1. Austyn JM. The dendritic cell system and anti-tumor immunity. In Vivo 1993;7:193-01
2. Bennaceur K, Chapman J, Brikci-Nigassa L, Sanhadji K, Touraine JL, Portoukalian J. Dendritic cells dysfunction in tumor environment. Cancer Lett 2008;272:186-96.
3. Binmadi, NO, Basile JR. Perineural invasion in oral squamous cell carcinoma: A discussion of significance and review of the literature Oral Oncol 2011; 47: 1005–1010.
4. Boshoff C, Weiss R. Cancer processes in immunodeficient populations: an introduction. Eur J Cancer. 2001; 37(10):1202-8.
5. Bova RJ, Quinn DI, Nankervis JS, et al. Cyclin D1 and p16 expression predict reduced survival in carcinoma of the anterior tongue. Clin Cancer Res 1999; 5:2810-9.
6. Carlos De Vicente J, Herrero-Zapatero A, Fresno MF, Lopez-Arranz JS. Expression of cyclin D1 and Ki-67 in squamous cell carcinoma of the oral cavity: clinicopathological and prognostic significance. Oral Oncol 2002; 38:301-8.
7. Carvalho AL, Ikeda MK, Magrin J, Kowalski LP. Trends of oral and oropharyngeal cancer survival over five decades in 3267 patients treated in a single institution. Oral Oncol 2004;40(1): 71–6
8. Chung CH, Bagheri A, D'Souza G. Epidemiology of oral human papillomavirus infection. Oral Oncol (2013), in press.
9. Cortesina G, Martone T. Molecular metástases markers in head and neck squamous cell carcinoma: review of the literature. Acta Otorhinolaryngol Ital 2006; 26:317-25
10. Curado MP, Johnson NW, Kerr AR, Silva DRME, Lanfranchi H, Pereira DL, Fonseca, FP, Lopes, MA, Kowalski LP, Vargas PA. Oral and oropharynx cancer in South America: Incidence, mortality trends and gaps in public databases as presented to the Global Oral Cancer Forum. Translational Research Oral Oncol 2016; **1**: 1-7
11. Fagan JJ, Collins B, Barnes L, D'Amico F, Myers EN, Johnson JT. Perineural Invasion in squamous cell carcinoma of the head and neck. Arch Otolaryngol Neck Surg 1998;124(6):633–7.
12. Folkman, J. Angiogenesis in câncer, vascular, rheumatoid and other disease. Nat Med 1995; 1: 27-31.

13. Gakiopoulou H, Korkolopoulou P, Levidou G, Thymara I, Saetta A, Piperi C, Givalos N, *et al*. Minichromosome maintenance proteins 2 and 5 in non-benign epithelial ovarian tumours: relationship with cell cycle regulators and prognostic implications. Br J Cancer. 2007; 97(8):1124-34

* De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Committee of Medical Journal Editor – Grupo Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

14. Goldman SA, Baker E, Weyant RJ, Clarke MR. et al. Peritumoral CD1apositive dendritic cells are associated with improved survival in patients with tongue carcinoma. Arch Otolaryngol Head Neck Surg, v.124, p. 641-1998.

15. Gomes JO, Carvalho MV, Fonseca FP, Gondak RO, Lopes MA, Vargas PA. CD1a+ and CD83+ Langerhans cells are reduced in lower lip squamous cell carcinoma. J Oral Pathol Med 2016; **45**: 433–439.

16. Hu M, Li K, Maskey N, et al. Decreased intratumoral Foxp3 Tregs and increased dendritic cell density by neoadjuvant chemotherapy associated with favorable prognosis in advanced gastric cancer. Int J Clin Exp Pathol 2014; 7: 4685–94.

17. Iwamoto M, Shinohara H, Miyamoto A, et al. Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. Int J Cancer 2003; 104: 92–7.

18. Jones HB, Sykes A, Bayman N, Sloan P, Swindell R, Patel M, Musgrove B. The impact of lymphovascular invasion on survival in oral carcinoma. Oral Oncol 2009; 45: 10-15.

19. Kato H, Miyazaki T, Fukai Y, et al. A new proliferation marker, minichromosome maintenance protein 2, is associated with tumor aggressiveness in esophageal squamous cell carcinoma. J Surg Oncol 2003; 84:24-30.

20. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology 2007;**121**(1):1-14.

21. Kodani I, Shomori K, Osaki M, Kuratate I, Ryoke K, Ito H. Expression of minichromosome maintenance 2 (MCM2), Ki-67, and cell cycle related molecules, and apoptosis in the normal dysplasia sequence of the oral mucosa. Pathobiology 2001; 69:150-8.

22. Kowalski LP, Magrin J, Waksman JS, Santo GF, Lopes ME. Supraomohyoid neck dissection in the treatment of head and neck tumors. Survival results in 212 cases. Arch Otolaryngol Head Neck Surg. 1993; 119 (9): 958-63.

23. Kowalski LP, Sanabria A. Elective neck dissection in oral carcinoma: a critical review of the evidence. Acta Otorhinolaryngol Ital. 2007; 27:113-117.

24. Kowalski LP. Head and neck cancer. BMC Proceedings 2013; 7(Suppl 2):K12

25. Kruse AL, Bredell M, Grätz KW. Oral squamous cell carcinoma in nonsmoking and non-drinking patients. Head Neck Oncol 2010 2:24.

26. Liebig C, Ayala G, Wilks J, Verstovsek G, Liu H, Agarwal N, et al. Perineural invasion is an independent predictor of outcome in colorectal cancer. J Clin Oncol 2009;27:5131–36

27. Marioni G, Marino F, Giacomelli L, et al. Endoglin expression is associated with poor oncologic outcome in oral and oropharyngeal carcinoma. Acta Otolaryngol 2006; **126**: 633–9

28. Martone T, Rosso P, Albera R, et al. Prognostic relevance of CD105 + microvessel density in HNSCC patient outcome. Oral Oncol 2005; **41**: 147–55.

29. Massano J, Ragateiro FS, Januário G, Ferreira A. Oral squamous cell carcinoma: review of prognostic and predictive factors. Oral Surg Oral Med Oral Pathol Oral Radiol End 2006; 102:67-76.

30. Mineta H, Miura K, Takebayashi S, Ueda Y, Misawa K, Harada H, Wennerberg J, Dictor M. Cyclin D1 overexpression correlates with poor prognosis in patients with tongue squamous cell carcinoma. Oral Oncol 2000; 36:194-198.

31. Ministério da Saúde. Instituto Nacional de Câncer. Estimativa 2014: incidência de câncer no Brasil. Rio de Janeiro: INCA, 2014.

32. Myoung H, Kim MJ, Lee JH, Ok YJ, Paeng JY, Yun PY. Correlation of proliferative markers (Ki-67 and PCNA) with survival and lymph node metastasis in oral squamous cell carcinoma: a clinical and histopathological analysis of 113 patients. Int J Oral Maxillofac Surg 2006; 35:1005-10.

33. Nagpal JK, Das BR. Oral cancer: reviewing the present understanding of its molecular mechanism and exploring the future directions for its effective management. Oral Oncol 2003; 39(3):213-21.

34. Peltonen JK, Helppi HM, Pääkkö P, Turpeenniemi-Hujanen H, Vähäkangas KH. P53 in head and neck cancer: Functional consequences and environmental implications of TP53 mutations. Head Neck Oncol 2010; 2-36.

35. Perez L, Shurin MR, Collins B, Kogan D, Tourkova IL, Shurin GV. Comparative analysis of CD1a, S-100, CD83, and CD11c human dendritic cells in normal, premalignant, and malignant tissues. Histol Histopathol 2005**; 20**: 1165–72.

36. Pich A, Chiusa L, Navone R. Prognostic relevance of cell proliferation in head and neck tumors. Ann of Oncol 2004; 15:1319-1329

37. Sarkis SA, Abdullah BH, Majeed BAA, Talabani NG. Immunohistochemical expression of epidermal growth factor receptor (EGFR) in oral squamous cell carcinoma in relation to proliferation, apoptosis, angiogenesis and lymphangiogenesis. Head Neck Oncol 2010; 2:13.

38. Scully C. Medicina oral e maxilofacial: bases do diagnóstico e tratamento. Rio de Janeiro: Elsevier; 2009. Câncer; p.177-90.

39. Schimming R, Marme` D. Endoglin (CD105) expression in squamous cell carcinoma of the oral cavity. Head Neck 2002; **24**: 151–6.

40. Silva SD, Perez DE, Alves FA, Nishimoto IN, Pinto CAL, Kowalski LP, Graner. ErB2 and fatty acid synthase (FAS) expression in 102 squamous cell carcinomas of the tongue: correlation with clinical outcomes. Oral Oncol 2008; 44:484-90

41. Sobin LH, Wittekind C. UICC, International Union Against Cancer, TNM classification of malignant tumours. 6th ed. New York: Wiley-Liss; 2002.

42. Soudry E, Preis M, Hod R, Hamzany Y, Hadar T, Bahar G, et al. Squamous cell carcinoma of the oral tongue in patients younger than 30 years: clinicopathologic features and outcome. Clin Otolaryngol 2010;35:307–12.

43. Speight PM, Barrett AW. Prognostic factors in malignant tumours of the salivar glands. Br J Oral Maxillofac Surg 2009;47:587–93.

44. Takes RP, Rinaldo A, Silver CE, Haigentz M Jr, Woolgar JA, Triantafyllou A, *et al*.Distant metastases from head and neck squamous cell carcinoma. Part I. Basic aspects. Oral Oncol. 2012 Sep;48(9):775-9

45. Todd R, Hinds PW, Munger K, et al. Cell cycle dysregulation in oral cancer. Crit Rev Oral Biol Med 2002;13:51-61.

46. Upadhyay RB, Upadhyay J, Rao NN, Agrawal P. The role of Langerhans cells in oral squamous cell carcinoma. Chin Ger J Clin Oncol 2011; **10**: 606-11

47. van Monsjou HS, Wreesmann VB, van den Brekel MW, Balm AJ. Head and neck squamous cell carcinoma in young patients. Oral oncol. 2013;49(12):1097-102.

48. Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009; 45(4-5):309-16.

49. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis correlation in invasive breast carcinoma. N Engl J Med 1991; **324**:1–8.

50. Woolgar JA. Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. Oral Oncol 2006;42:229-39.

51. Wright-Browne V, McClain KL, Talpaz M, Ordonez N, Estrov Z. Physiology and pathophysiology of dendritic cells. Hum Pathol 1997; 28:563-579

52. Xie X, De Angelis P, Clausen OP, Boysen M. Prognostic significance of proliferative and apoptotic markers in oral tongue squamous cell carcinomas. Oral Oncol 1999; 35:502.

53. Yilmaz T, Gedikoglu G, Celik A, Onerci M, Turan E. Prognostic significance of Langerhans cell infiltration in cancer of the larynx. Otolaryngol Head Neck Surg. 2005 ;132(2):309-16.

54. Yu M, Liu L, Liang C, Li P, Ma X, Zhang Q, Wei Y. Intratumoral vessel density as prognostic factors in head and neck squamous cell carcinoma: A meta-analysis of literature. Head Neck 2014; **36**: 596-602.

55. Zhao D, Pan J, Li XQ, Wang XY, Tang C, Xuan M. Intratumoral lymphangiogenesis in oral squamous cell carcinoma and its clinicopathological significance. J Oral Pathol Med 2008; **37**: 616-25

ANEXOS

Anexo 1 – folha de aprovação do comitê de ética

Hospital A.C.Camargo
Centro de Tratamento, Ensino e Pesquisa em Câncer

Comitê de Ética em Pesquisa - CEP

São Paulo, 15 de Agosto de 2012.

Ao
Dr. Luiz Paulo Kowalski

Ref.: Projeto de Pesquisa nº. 1684/12
"Análise Clinicopatológica da angiogênese e linfagiogênese como fator prognóstico em carcinomas espinocelulares de boca em estádio clínico avançado III e IV".

Os membros do Comitê de Ética em Pesquisa em Seres Humanos da Fundação Antonio Prudente – Hospital do Câncer - A.C. Camargo/SP, em sua última reunião de 14/08/2012, **aprovaram** a realização do projeto do estudo em referência e tomaram conhecimento dos seguintes documentos:

- Folha de Rosto para Pesquisa Envolvendo Seres Humanos;
- Termo de Compromisso do Pesquisador com as Resoluções do Conselho Nacional de Saúde;
- Termo de Dispensa do Consentimento Livre e Esclarecido;
- Declaração sobre os Dados Coletados, Publicação dos Dados e Propriedade das Informações Geradas;
- Declaração Sobre o Uso e Destino do Material Biológico, Publicação dos Dados e Propriedades das Informações Geradas;
- Orçamento Financeiro Detalhado;
- Declaração de Infraestrutura e Instalações do Departamento de Cirurgia de Cabeça e Pescoço e Otorrinolaringologia;
- Declaração de Ciência e Comprometimento do Departamento de Cirurgia de Cabeça e Pescoço e Otorrinolaringologia;
- Declaração de Ciência e Comprometimento do Departamento de Anatomia Patológica.

Informações a respeito do andamento do referido projeto deverão ser encaminhadas à assistente do CEP dentro de 12 meses.

Atenciosamente,

Dr. Jefferson Luiz Gross
1º Vice-Coordenador do Comitê de Ética em Pesquisa

1/1

Fundação Antonio Prudente – CNPJ/MF N. 60.961.968/0001-06
Rua Prof. Antônio Prudente, 211 – Liberdade – São Paulo, SP – 01509-900
Telefone: (11) 2189-5000
www.accamargo.org.br

A.C.Camargo Cancer Center

Comitê de Ética em Pesquisa - CEP

São Paulo, 23 de setembro de 2013.

Ao
Prof. Dr. Luiz Paulo Kowalski.

Ref.: Projeto de Pesquisa nº. 1684/12
"Análise Clinicopatológica da angiogênese e linfagiogênese como fator prognóstico em carcinomas espinocelulares de boca em estádio clínico avançado III e IV".

Os membros do Comitê de Ética em Pesquisa em Seres Humanos da Fundação Antonio Prudente – Hospital do Câncer - A.C. Camargo/SP, em sua última reunião de 17/09/2013, **tomaram conhecimento** do seguinte documento:

- Inclusão da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como instituição coparticipante do projeto em referência para inclusão do aluno de Mestrado Juscelino de Freitas Jardim da mesma instituição, sob orientação do Prof. Dr. Luiz Paulo Kowalski, em carta datada de 27 de agosto de 2013;
- Relatório de Acompanhamento do estudo em referência, datado de 27 de agosto de 2013.

Atenciosamente,

Dr. Jefferson Luiz Gross
1º Vice-Coordenador do Comitê de Ética em Pesquisa

Anexo 2 - ficha para coleta de dados

Hospital do Câncer AC Camargo – Fundação Antônio Prudente
Departamento de Cirurgia de Cabeça e Pescoço
Dr. Luiz Paulo Kowalski e Juscelino Freitas
CARCINOMA ESPINOCELULAR T3 E T4

1-Registro Hospitalar.....(_____)
2-Idade:_____(anos).....(_____)
3-Sexo: (1)Masculino (2)Feminino.....(____)
4-Grupo étnico: (1)Branco (2)Negro (3)Amarelo (4)Pardo (5)Outro.....(____)
5-Tempo de queixa (meses):.....(_____)
6-Tipo de queixas:
6a)Dor: (0)Não (1)Sim.....(____)
6b)Tumor: (0)Não (1)Sim.....(____)
6c)Outras: (0)Não (1)Sim.....(____)
7-Localização: (1) Língua (2) Soalho bucal .....(____)
8-Lateralidade: (1)Unilateral (2)Bilateral (3)Mediana.....(____)
9-Biópsia prévia: (0)Não (1)Incisional (2)Agulha (3)Outra.....(____)
10-Tipo Histológico:.....(____)
11-Grau CME: (1)Baixo (2)Intermediário (3)Alto.....(____)
13-Maior diâmetro do tumor (cm):.....(_____)
14-Invasão estruturas adjacentes:(0)Não (1)Língua (2) Soalho (3) Gengiva (4) Músculo (5) Pele (6) Outras.....(____) _____
15- Números de Linfonodos comprometidos clinicamente.....(____)
16-Local das recidivas: Ipsilaterais níveis (0) Não (1) I (2) II (3) III (4) IV (5) V .....(____)
Local das recidivas à distância _____
17-Estádio Clínico (TNM):
Critério T: (1)T1 (2)T2 (3)T3 (4)T4a (5)T4b (6)Tx.....(____)
Critério N: (0)N0 (1)N1 (2)N2a (3)N2b (4)N2c (5)N3 (6)Nx.....(____)
Critério M: (0)M0 (1)M1 (2)Mx.....(____)
18-Estadiamento: (1)III (2) IVa (3)IVb (4)IVc.....(____)
19-Metástases à distância ao diagnóstico:.....(____)
(0)M0 (1)Pulmão (2)Osso (3)Fígado (4)Cérebro (5)Outros
20-Data do início do tratamento:.....(____/____/____)
21-Seqüência de tratamento: (0)Não (1)Cirurgia (2)RXT (3)QT.....(____)

22-Tipo de cirurgia: (1) Glossectomia parcial (2) Hemiglossectomia (3) PG (Pelviglossectomia) (4) PGM (Pelviglossomandibulectomia marginal) (5) PGM seccional (6) Comando (7) Glossectomia total (8) Glossectomia total com mandibulectomia .....( )

23-Esvaziamento cervical:.....(____)

(0)Não (1)SHOuni (2)Radical (3)Radical modificado (4)Outro

24-Complicaçãoes:.....(____)

(0)Não (1) Deiscência/ Necrose do retalho (2)Infecção (3)Seroma (4) hematoma (5)Fístula (6) Outra _____

25-Metástase em linfonodos após avaliação: (0)Não (1)Sim.....(____)

26- Margens: (0) Livres (1) Exíguas (<5mm) (2) Comprometidas .....(____)

27- Invasão vascular: (0) Não (1) Sim .....(____)

28 – Invasão Perineural: (0) Não (1) Sim .....(____)

29 – Número de linfonodos Ipsilaterais: _____

30 – Número de linfonodos Ipsilaterais: _____

31 – Imunoístoquimica: Marcadores _____

32-Data do inicio da RXT:.....(____/____/____)

33 Dose local (cGy):.....(_____)

34 -Dose cervical (cGy):.....(_____)

35-Recidiva: (0)Não (1)Sim.....(____)

36-Se recidiva regional: (1)Ipsilateral (2)Contralateral (3)Bilateral .....(____)

37-Se recidiva à distância: (1)Pulmão (2)Fígado (3)Osso (4)Cérebro (5)Outro.....(____)

38-Data da primeira recidiva:.....(____/____/____)

39-Tratamento da recidiva:.....(____)

(1)Ressecção local (2)Ressecção recidiva cervical (3)RXT (4)Esvaziamento cervical (5)QT (6)Outra .....( )

40- Número do Anátomo patológico:.....(_____)

41-Data da última informação: .....(____/____/____)

42- Situação da última informação: .....(____)

(1)Vivo sem doença (2)Vivo com doença (3)Morto pela doença (4)Morto por outras causas (5)Perdido de vista